Differential regulation of monocyte cytokine release

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To Donna for her support, patience and inspiration, and my parents for making it possible for me to pursue my ambitions.

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CONTENTS

SUMMARY

The regulation of monocyte cytokine production is critical to the pathogenesis of diseases such as rheumatoid arthritis (RA). In particular, the balance between the production of pro- and anti-inflammatory cytokines by mononuclear phagocytes within the rheumatoid synovium may modulate tissue degradation and joint destruction.

The studies of this thesis have shown that monocyte production of TNFα, IL-6, OSM, IL-1β and IL-1ra are differentially regulated by intracellular cation levels. Inhibition of the Na^+/K^+-ATPase, which in the synovium may be due to oxidative damage, has been shown to greatly contribute to the generation of a pro-inflammatory cytokine profile from stimulated monocytic cells. The specific Na^+/K^+-ATPase inhibitor ouabain has been shown to increase IL-1β processing in monocytes stimulated with LPS, or viable activated T cells, and to concurrently down-regulate IL-1ra production. The mechanism of action of ouabain in these models may well involve increased activation of ICE, due to K^+ efflux and indeed it has been shown that increased concentrations of ouabain can induce both apoptosis and necrosis in monocytes

The ability of T cells to regulate production of IL-1β and IL-1ra from neighbouring monocytes may be an important mechanism in the pathogenesis of RA. The models studied here have shown that fixed T cells were able to induce the secretion of both IL-1β and IL-1ra from resting monocytes, perhaps due to surface CD69 expression, but that the balance in cytokine production was not consistently driven in a pro- or anti-inflammatory direction. Monocytes stimulated with viable T cells, however, produced a markedly increased amount of IL-1β, implying that T cell derived soluble factors may be more important in driving a pro-inflammatory cytokine response from monocytes. The balance between production of IL-1β and IL-1ra from monocytes has also been shown to be differentially regulated by FcR aggregation and LPS stimulation. The pathways that are specific to signals induced by these stimuli remain to be determined but evidence here supports the involvement of PI3-K in T cell-, IgG- and LPS- induced IL-1ra but not IL-1β production.

Abbreviations

A23187	Calcium ionophore
APPs	Acute phase proteins
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
Ca^{2+}	Calcium ions
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CHO cells	Chinese hamster ovary cells
CRP	C reactive protein
CSF	Colony stimulating factor
CTL	Cytotoxic T lymphocyte
DMEM	Dulbecco's Modified Eagles Medium
EBV	Epstein Barr virus
ECACC	European collection of animal cell cultures
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (b-amino-ethyl ether) N, N, N', N', tetraacetic acid
ELISA	Enzyme linked immunosorbant assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FSC	Forward scatter
GAG	Glycosaminoglycan
GM-CSF	Granulocyte/macrophage colony stimulating factor
H_2O_2	hydrogen peroxide
HRP	Horse radish peroxidase
ICE	Interleukin one converting enzyme
ICEi	Interleukin one converting enzyme inhibitor
IFNγ	Interferon gamma
Ig(G, M, A, E)	Immunoglobulin (G, M, A, E)
IL	Interleukin
IL-1ra	Interleukin one receptor antagonist
IL-1RI/RII	Interleukin one receptor type I and II
IP3	Inositol 1, 4, 5-trisphosphate
K^+	Potassium ions
LPS	Lypopolysaccharide (endotoxin)
MFI	Mean fluorescence intensity
Mg^{2+}	Magnesium ions
MHC	Major histocompatibility complex
MIF	Monocyte migration inhibitory factor
mRNA	Messenger ribonucleic acid
Na^+	Sodium ions
Na^+/H^+ exchanger	Sodium/proton exchanger
Na^+/Ca^{2+} exchanger	Sodium/calcium exchanger
$Na^+/K^+/2Cl^-$	Sodium/potassium/chloride cotransporter
NADP/H	Nicotinimide adenine dinucleotide phosphate/reduced
Na^+/K^+-ATPase	Sodium/potassium adenosine triphosphatase (sodium pump)
NSAIDs	Non-steroidal anti-inflammatory drugs
OA	Osteoarthritis

OPD	Ortho phenyl diamine
PBMC	Peripheral blood mononuclear cell
PDBu	Phorbol dibutyrate
PDGF	Platelet derived growth factor
PG	Proteoglycan
PGE_2	Prostaglandin E2
PHA	Phytohaeagglutinin
PKC	Protein kinase C
PMA	Phorbol Myristole acetate
PMN	Polymorphonuclear cells
Pro-IL1	IL-1 precursor protein
PsA	Psoriatic arthritis
RA	Rheumatoid arthritis
RF	Rheumatoid factor
ROI	Reactive oxygen intermediates
SSC	Side scatter
TE	Trypsin EDTA
TMB	Tetramethyl benzadine
TNFα	Tumour necrosis factor α

CHAPTER 1

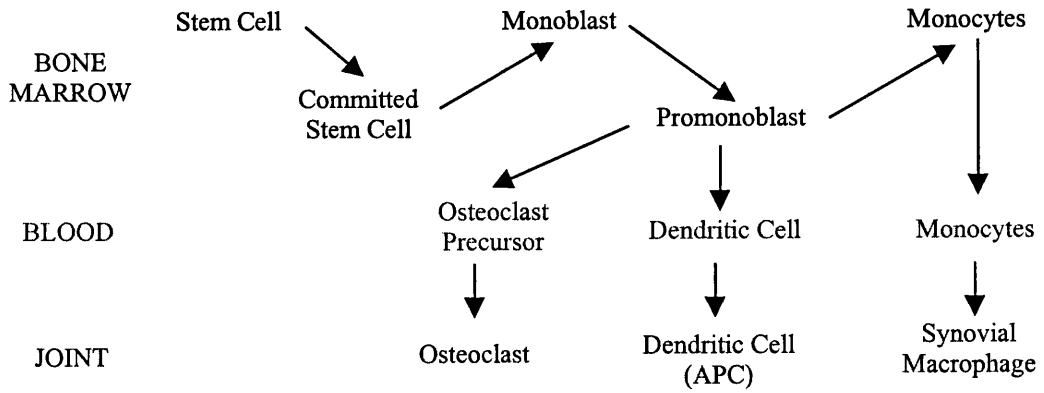
INTRODUCTION

1: INTRODUCTION

This thesis will examine the differential regulation of monocyte-derived cytokines and explore pathways of monocyte activation that may be important in the pathogenesis of inflammatory diseases such as rheumatoid arthritis (RA). It is now more apparent than ever that the outcome of an immune response is not controlled by single cytokine signals but the complex orchestration of multiple protein mediators. With time, more and more evidence is being gathered to show multiple functions for many cytokines, with some clearly having both pro- and anti-inflammatory roles in the immune response. Amidst this confusion, however, at the end-point of a misdirected immune response, certain monocyte-derived cytokines have been clearly identified as being instrumental in the tissue damage and destruction caused in RA. It is these mediators that are studied here and this introduction will endeavour to detail the current knowledge of their regulation and roles in RA.

1.1: The Monocyte-Macrophage Cell lineage

Originally, monocytes and macrophages were classified as cells of the reticulo-endothelial system. Van Furth et al. (1972) (1), however, proposed the mononuclear phagocyte system, and monocytes and macrophages became basic cell types of this system. Their development takes place in the bone marrow and passes through the following steps:



Stem cells committed to differentiation into mononuclear phagocytes pass through at least two readily identifiable morphological steps in the bone marrow, namely monoblasts and pro-monocytes. These cells undergo a series of programmed

divisions to form monocytes, which after release from the bone marrow into blood, circulate for one to three days before migrating into various extravascular compartments. Monocytes do not undergo further division after entering the bloodstream. Rather, their emigration into various tissues and organs is marked by pronounced morphological and functional differentiation, sometimes characteristic of their location. Mononuclear phagocytes are extremely pleiotypic in that they can undergo a complex series of differentiation processes in order to adapt to a particular environment, e.g. osteoclasts in bone and Kupffer cells in the liver, and be receptive to multiple signals from other elements of the host defence system.

Under steady-state conditions of health, traffic of monocytes in and out of the circulation occurs at a relatively slow rate. The turnover of mononuclear phagocytes increases rapidly when the host encounters an inflammatory stimulus or infectious agent. Under these conditions humoral factors are generated which stimulate the formation of monocytes in the bone marrow and their release into the bloodstream with accumulation at the sites of inflammation and infection occurring within twelve to twenty-four hours after the initial influx of neutrophils. The newly recruited monocytes undergo rapid differentiation to facilitate the recognition and removal of the inflammatory or infectious stimuli.

Once monocytic cells have entered the inflamed tissue it is believed that they do not re-circulate. Under conditions of inflammation, therefore, inflammatory mediators present in the joint may induce local differentiation and activation of the invading monocytes causing increased inflammatory responses and the formation of granulomas. In RA granulomas consisting of epitheloid and giant cells are often found in the synovial tissues and also in rheumatoid nodules.

Cytokines play critical roles in controlling the differentiation of the monocyte/macrophage system. For example, TNFα inhibits differentiation of monocytes into macrophages and, together with granulocyte-macrophage colony stimulating factor (GM-CSF), directs the precursor cells to differentiate into dendritic cells (2-4), which are potent professional antigen-presenting cells (5,6). Another branch of the monocyte differentiation system is the formation of osteoclasts, which greatly contribute to bone destruction in RA (7).

The presentation of (auto) antigens to synovial T cells could be a major mechanism by which cells of the macrophage/monocyte family contribute to the perpetuation of

inflammatory joint disease. Class II MHC-positive antigen presenting cells may be involved in central initiation of auto-immunity due to their ability to educate T cell responses in the thymus in the development of self-tolerance (8). Also, there is evidence that mononuclear phagocytes are capable of presenting antigen in the synovial membrane. Besides the expression of class II MHC molecules, molecules such as CD40, CD54, CD80 and CD86 (9-11) , and intracellular adhesion molecule 3 (12) have been detected on synovial monocytes/macrophages. These molecules exert important cognate functions in antigen recognition, defined as signal 2, without which T cell anergy occurs.

The significance of antigen presentation in the rheumatoid synovium has been questioned due to the lack of unequivocal evidence of oligoclonal T cell expansion and the failure to detect definitive candidate auto-antigens. On the other hand, it is not clear which cell is most relevant for the processing of a putative antigen, since synovial fibroblasts and chondrocytes are also capable of presenting antigen in vitro (13,14). In addition, the specific accumulation of well-differentiated dendritic cells in rheumatoid synovium has been recently confirmed (15). Various candidate auto-antigens have been proposed in RA, including heat shock proteins, cartilage link protein and other proteoglycans (reviewed by Moots and Wucherpfennig (16)). The most compelling evidence supports the role of type II collagen as a possible auto-antigen in RA and studies have shown that T cells reactive to type II collagen may exist in patients with RA (17).

The precise role played by monocyte/macrophages in the induction of RA remains an area of debate. One thing that is certain, however, is that monocyte derived cytokines dominate the cytokine profile in inflammatory synovitis. In RA, activated macrophages are enriched in the synovium and are also found in strategic sites within the destructive pannus tissue (18,19). In addition, macrophages and circulating monocytes secrete large quantities of prostanoids and cytokines (20-22), and peripheral blood monocytes show increased phagocytic activity (23). The expression of CD14, FcγRI and FcγRII have been shown to be upregulated on the surface of peripheral blood monocytes from patients with active RA. Interestingly, FcγRI and FcγRII expression on monocytes from RA patients who were in complete remission were still seen to be upregulated whereas CD14 expression had dropped (24).

To understand the role played by monocytes in the pathogenesis of RA a brief description of the disease will follow.

1.2: Rheumatoid Arthritis

Rheumatoid arthritis is predominantly an articular inflammatory disease with considerable evidence of abnormal mononuclear cell activity. The inappropriately self-directed (autoimmune) inflammatory response results in chronic synovitis, infiltration, accumulation and persistent activation of lymphocytes and mononuclear phagocytes, which leads to subsequent tissue damage. Breakdown of articular cartilage and eventual bone erosion results in crippling deformation of the synovial joints. Although the most apparent pathology of RA is seen throughout the synovial joint other major organs are affected and systemic features include vasculitis, nodule formation and splenomegaly.

The aetiology of RA remains obscure but may involve the inappropriate recognition of endogenous self-antigens, such as collagen, mucopolysaccharides and rheumatoid factors or exogenous agents such as mycoplasma, mycobacteria and viruses. Interestingly, there is a threefold predominance of RA in females, which may indicate the involvement of hormonal factors, and there is also a clear genetic contribution to the disease contained within the HLA class II locus. There is a shared epitope on one side of the peptide binding groove of HLA-DR, comprised of the amino acids 70-74 of the β chain, which is conserved between the disease susceptible DR1 and DR4 haplotypes (25). Greater than 80% of Caucasian RA patient's display this epitope supporting the concept that T lymphocyte antigen recognition is an important factor at some stage in the pathogenesis of the disease. However, the involvement of non-genetic factors in RA pathogenesis is undisputed due to the discordance in the development of RA in identical twins (26).

Most patients with RA have circulating soluble materials with the characteristics of immune complexes and the existence of autoantibodies and immune complexes in RA has been recognised for several decades (27,28). Anti-γ-globulins of the IgG and IgM classes and IgG itself are integral parts of these soluble complexes. Whether these immune complexes are responsible for vasculitis or for the other extra-articular features or are merely markers for severe disease (29) remains a question for debate.

The rheumatoid synovium makes and contains a large amount of immunoglobulins (Ig's) although relatively few plasma cells are present. Plasma cells in the subsynovium primarily produce IgG and 20% of the IgG in the synovial fluid is produced in the synovium (reviewed by Maini 1987 (30)). The majority of the IgG in the synovial fluid is anti-IgG. In most cases the majority of IgM present in the synovium is rheumatoid factor (RF). Rheumatoid factors are defined as antibodies specific to antigenic determinants on the Fc fragments of human or animal immunoglobulin G. In RA the increased Ig production could be a result of B cell hyperreactivity due to excessive helper T cell stimulation or due to insufficient inhibition by suppressor T cells, either as a result of decreased numbers or compartmental exclusion. The IgG-anti-IgG produced by plasma cells form self associated intermediate sized complexes which go on to activate the complement cascade. These complexes can further stabilise by associating with IgM-RFs and other moieties such as anti-f(ab)'$_2$, anti-fibrinogen and collagen-anti-collagen complexes. Rheumatoid factors have often been linked with more severe disease, sero-negative patients often having characteristics of juvenile RA and more asymmetric joint involvement (31,32). In sero-positive RA increased severity of disease is also linked to HLA DR4 (33), HLA DR1 (34) or both.

1.2.1: Pathogenesis of RA

The inflammatory response seen in RA is characterised by vasodilatation induced by inflammatory mediators such as PGE_2, PGI_2, the complement component C3a, substance P and calcitonin gene-related peptide (CGRP) from sensory C fibres and nitric oxide (NO). Endothelial cell damage and increased expression of cell surface adhesion molecules such as ICAM-1 and ICAM-2 combined with the release of chemotactic factors such as LTB_4, IL-8 and C5a leads to vascular leakage and oedema. MCP-1 and MIP-1α have been identified as likely C-C chemokines responsible for the recruitment of mononuclear cells into the inflamed synovium (35-37). Immunoreactive MCP-1 and MIP-1α were found to be significantly elevated in both synovial fluid and serum of patients with RA. Immunolocalisation showed that both synovial tissue fibroblasts and macrophages constitutively expressed MCP-1 and MIP-1α. Both chemokines were expressed by cells of the macrophage-rich synovial lining layer from patients with RA, whereas normal synovial tissue

displayed little expression. Macrophages within the subsynovial lining of patients with RA were also highly immunopositive for both MCP-1 and MIP-1α. Additional studies demonstrated that cultured synovial fibroblasts constitutively expressed MCP-1, which was markedly enhanced by the addition of IL-1, IL-4 or IFNγ (38). The chemotactic gradients generated favour the infiltration of PMNs initially but changes in endothelial adhesion molecule expression and mononuclear cell surface integrin expression then favour the infiltration of mononuclear cells. Accumulating monocyte/macrophages maintain the inflammatory reaction due to the release of powerful pro-inflammatory cytokines such as IL-1β and TNFα. The normally acellular synovial fluid becomes predominantly enriched with neutrophils, but T lymphocytes, mononuclear phagocytes and dendritic cells are also found. The increased accumulation of cells is most obvious in the synovial membrane, where an increase from 1-2 cells to 6-8 cells is seen in the thickness of the joint lining layer, with activated resident monocyte/macrophages (often referred to as type A synoviocytes) and T cells predominating. Plasma cells, dendritic cells and activated fibroblasts are also found in abundance in the inflamed synovium and the cell surface phenotypes found favour antigen presentation. Synovial hyperplasia and pannus formation, which is characterised by extending villi of inflamed synovium that intrude upon the articular cartilage, involves many cytokines and growth factors provided by the ongoing inflammatory reaction within the joint, and it is at this junction between the pannus and the articular cartilage that the most tissue destruction occurs. The type A synoviocytes, present in the invading pannus, have numerous lysosomes and vacuoles with relatively small amounts of endoplasmic reticulum, resembling monocyte/macrophage-like cells that are active in both phagocytosis and secretion of proteolytic enzymes. Activated neutrophils and macrophages that infiltrate the joint release enzymes that lead to cartilage destruction. Inappropriate activation of osteoclasts can then lead to destruction of the bone (39).

The destruction of cartilage seen in RA is now considered to be mostly due to the activity of matrix metalloproteinases (MMP's), enzymes produced by activated macrophages and fibroblasts in response to pro-inflammatory cytokines such as IL-1 and TNFα. These enzymes are synthesised and secreted as latent molecules, with activation induced by the proteolytic cleavage of a regulating pro-peptide domain. In RA two of the principal MMP's involved, whose production has been shown to be

increased, are collagenase (MMP-1) and stromelysin (MMP-3) (40). The tissue inhibitors of metalloproteinases (TIMP's), that irreversibly bind the active MMP forming an inactive heterodimer provide further regulation of the MMPs. Interestingly, TIMPs are produced by the same cells that produce MMPs indicating the fine balance that their regulation must depend upon. The competition between the production of MMPs and TIMPs could be critical in the regulation of matrix turnover and the outcome of diseases such as RA. It has been shown that certain immunomodulatory and anti-inflammatory cytokines such as transforming growth factor β (TGFβ) and IL-10, not only depress the production of certain pro-inflammatory cytokines that induce MMPs, but also induce the production of TIMPs (41).

Rheumatoid synovitis is thus characterised by an exudative phase, involving the microcirculation and lining cells of the synovium, that allows an influx of plasma proteins and cellular elements into the joint and a chronic inflammatory phase featuring mononuclear cell infiltration into the subsynovium. The exact role of T lymphocytes and monocyte/macrophages in the initiation and progression of synovitis remains somewhat unclear (42,43). The involvement of monocyte derived cytokines, such as IL-1, granulocyte/macrophage-colony stimulating factor (GM-CSF), IL-8, platelet derived growth factor (PDGF), transforming growth factor α (TGFα), TGFβ, IL-6 and TNFα, however is undisputed. Local synthesis of IL-1α, IL-1β and TNFα in rheumatoid synovium as well as increased levels of these cytokines in the synovial fluid has been well documented in RA (22,44-46). These cytokines are just a few among the many that are important in regulating the immune response but their powerful pro-inflammatory activity and undoubted involvement in inflammatory disease make them the focus of much research.

1.2.2: Pro-inflammatory cytokines

Many pro-inflammatory cytokines are involved in the control of inflammatory reactions and the pathogenesis of chronic inflammation but perhaps the most relevant to RA are TNFα and IL-1β. Consequently, TNFα and IL-1β will be discussed in greater detail in following sections but here the intention is to introduce other pro-inflammatory cytokines that play a part in the pathogenesis of RA.

IL-1β and TNFα protein is readily detected in the rheumatoid synovial fluid (45,47-50). These cytokines can also be detected at the mRNA level in the synovial membrane using in situ hybridisation (22,51) and blotting (52). Immunohistochemical localisation of these proteins has demonstrated predominant expression in macrophages (22,44). These proteins were also detected in short-term *in vitro* cultures of enzymatically disaggregated synovial membrane samples (52,53). Of importance was the observation that IL-1β and TNFα could be detected by bioassay of synovial membrane cultures, and hence were present in quantities able to signal effectively.

Subsequently, as other cytokine and growth factor cDNAs were cloned, their mRNAs and proteins were also detected in RA synovial tissue. These cytokines include IL-6 (54-57), interferon α (IFNα) (50), granulocyte macrophage colony stimulating factor (GM-CSF) (58-60), macrophage stimulating factor (M-CSF) (61), and leukocyte inhibitory factor (LIF) (62-64).

The growth factors identified as major contributors in the pannus formation and synovial hyperplasia seen in RA include platelet-derived growth factor (PDGF) (65), fibroblast growth factor (FGF) (65-67), and transforming growth factor β (TGFβ) (65,67-70).

1.2.3: Anti-inflammatory cytokines

Immunoregulatory and anti-inflammatory cytokines were generally discovered and cloned after the pro-inflammatory cytokines. These cytokines include IL-1receptor antagonist (IL-1ra), TGFβ, IL-4, IL-10 and IL-13. A number of studies from different groups have demonstrated that TGFβ is abundant in both the precursor inactive form and the active form in rheumatoid joints (68,71-74). Whether this cytokine functions as an anti-inflammatory mediator in RA, however, remains an area of debate. TGFβ can have pro-inflammatory effects such as acting as a monocyte chemoattractant (75) and causing synovial hyperplasia (67-70). On the other hand, TGFβ is likely to be a key cytokine that is involved in tissue repair and fibrosis in the joint. For example, whilst inhibiting production of MMPs such as collagenase (71) and inducing TIMP (41), TGFβ also stimulates the production of type I and type XI collagen (76).

However, whilst locally promoting tissue repair, overproduction of TGFβ in chronic lesions may lead to tissue destruction via recruiting inflammatory monocytes and fibroblasts and promoting synovial hyperplasia.

In common with TGFβ, IL-4 also displays some immunoregulatory effects such as inhibition of LPS-induced IL-1β, TNFα, PGE_2 and 92–kDa gelatinase production in human monocytes (68,77-79). However, IL-4 is not highly expressed in rheumatoid synovial tissue cultures (80) or in T cells cloned from RA synovial biopsies (81). The major source of IL-4 is T cells of the helper type two phenotype and the decreased expression of IL-4 in the rheumatoid synovium may be due to the predominance of T cells of the type 1 helper phenotype (82). IL-4 production may well be defective in RA joints and there is also a report showing that the incidence of allergies is lower in RA patients (83).

IL-10 also has profound anti-inflammatory effects. Its presence has been well documented in RA peripheral blood (84) and synovial joints (85,86). Blocking IL-10 activity with neutralising antibodies has been shown to enhance the production of IL-1β and TNFα in synovial cell cultures (85). Conversely, addition of IL-10 to these cultures inhibited production of IL-1β and TNFα by approximately 50%.

IL-1ra expression may also be relevant to the outcome of the inflammatory condition in RA. Along with decoy soluble IL-1 receptors, IL-1ra plays a key role in regulating the action of IL-1β. The expression of IL-1ra has been analysed in RA joints and both an upregulation of mRNA and protein has been observed in synovial fluid and joint cell cultures (87-90). The balance between IL-1ra and IL-1β production in RA however still favours the effects of IL-1β as levels of IL-1ra are insufficient to neutralise IL-1β bioactivity. IL-1ra does have high affinity for membrane type I and II IL-1 receptors but due to the ability of IL-1 to activate cells at very low receptor occupancy, a considerable molar excess (approx. 100:1) of IL-1ra is required to inhibit IL-1β effectively. Normal joint tissues express very little IL-1ra, so IL-1ra production is upregulated in the disease process but not sufficiently to neutralise IL-1. It is of interest that a study of the ratio of IL-1ra:IL-1β in synovial fluid of patients with Lyme arthritis indicated that the patients with the most favourable outcome had the highest ratio (91).

In this thesis the emphasis of the research is on the regulation of monocyte derived cytokines and as a result the next section will detail more about their roles in inflammation.

1.3: Monocyte-derived Cytokines

1.3.1: Interleukin 1 and its receptors

IL-1 was one of the first cytokines described. It was studied for many years for its ability to induce fever, stimulate hepatic acute-phase protein synthesis, augment lymphocyte responses, induce joint degeneration and cause proliferation of bone marrow cells. Although mainly studied as an element of host defence and disease there is also evidence for a role of IL-1 in normal health. For example, IL-1 expression is seen in the placental trophoblast and there may be a role for IL-1 in embryonic development, implantation and birth. However, studies carried out using gene deletions in mice have shown normal ovulation, fertilisation, implantation and parturition regardless of IL-1β, IL-1β converting enzyme and IL-1RI knockouts (reviewed in (92)). As a result it is no surprise that attention has focused on the role of IL-1 in disease. IL-1 is a potent inflammatory cytokine and the production and activation of IL-1, especially IL-1β, is tightly regulated. In addition to controlling gene expression, synthesis, and secretion, this regulation extends to surface receptor expression, soluble (decoy) receptors and a receptor antagonist protein (92).

The IL-1 gene family is composed of IL-1α, IL-1β and Il-1ra and is found, in humans, on the long arm of chromosome 2 (93). They are first synthesised as precursor proteins with pro-IL-1α and pro-IL-1β each having a molecular mass of 31kDa. Both pro-IL-1α and mature 17kDa IL-1α are biologically active (94) in contrast however, pro-IL-1β is relatively inactive and needs cleavage to a 17kDa peptide for optimal activity. The IL-1ra precursor has a leader sequence, is cleaved to its mature form and is secreted like most proteins.

The IL-1 receptor family is comprised of four members: IL-1RI, which is found in low numbers on nearly all cells; IL-1RII, which is primarily found on neutrophils, monocytes, and B-lymphocytes; IL-1 accessory protein (IL-1 AcP); and the T1/ST2/Fit-1 receptor. The gene for IL-1RI has no TATA box in its promoter and thus this receptor is constitutively expressed, falling into the category of the

housekeeper gene promoters (95). Various stimuli, such as PGE_2, phorbol ester, IL-1, IL-2 and IL-4 have been shown to increase the numbers of cell surface IL-1RI (96), but in some cell types IL-1 down regulates the number of its own receptors (97) by a decrease in mRNA half-life.

The IL-1RII has a cytosolic domain of only 29 amino acids and does not transduce a signal (98). The type II receptor may act as a decoy receptor, competing with the signal transducing type I receptor on the cell surface and also possibly as a shed receptor in the circulation. Soluble IL-1RII may serve as a natural antagonist to the effects of IL-1. It is worth noting however that very few IL-1RI need to be occupied for a committed biological effect due to a very efficient and greatly amplified signal transduction mechanism. IL-1 AcP has recently been shown to play a part in IL-1 signal transduction by increasing the affinity of IL-1RI for IL-1β (99-102). In general IL-1α and IL-1ra have a higher affinity for IL-1RI whereas IL-1β has a higher affinity for IL-1RII (97).

1.3.1.1: Genetic Regulation of IL-1

The genes for IL-1α, IL-1β and IL-1ra are differentially expressed (103,104) The promoter for IL-1α does not contain a TATA box but the IL-1β promoter does (104). The gene for IL-1ra is predominantly inducible in most cell types but the intracellular isoform of IL-1ra (icIL-1ra) is constitutively expressed in keratinocytes and intestinal epithelial cells (105). The gene encoding for IL-1β is not spontaneously expressed in mononuclear cells from the blood of healthy humans, but upon stimulation with bacterial endotoxin, mRNA for IL-1α and IL-1β is present within 15 minutes. IL-1β mRNA accumulates for 4 hours followed by a rapid fall that results in part from the synthesis of a transcriptional repressor as well as a decrease in mRNA half-life (106). The regulation of IL-1β transcription in monocytes has been most extensively studied in response to LPS (92,107,108). Several transcription factors have been identified including NF-IL6, cAMP-response element binding protein (109), NF–κB (110) and a STAT-like factor (111) that bind an upstream induction sequence. A promoter-proximal sequence has also been studied and shown to bind NF-IL6 (112,113) and a B-cell and myeloid-specific transcription factor PU.1 (114,115). Recent studies have also shown that IL-1β induced IL-1β mRNA involves the

induction of multiple protein complexes that incorporate the nuclear factor Oct-1 (116). In addition to transcriptional regulation, the stability of IL-1 mRNA can be selectively modulated by various stimuli (108).

Various other stimuli, such as blood clotting or complement components induce the synthesis of IL-1β mRNA in monocytic cells without significant translation into protein (117). Without translation most of the mRNA is degraded. This translational block, however, can be removed by stimulating the IL-1β-transcribing cells with bacterial endotoxin or IL-1 itself. Stabilisation of the AU-rich 3' untranslated region is thought to promote translation, but other mechanisms may be involved.

However, in contrast to IL-1β even under conditions of cell activation human monocytes do not readily process or secrete mature IL-1α (118,119). The lack of leader peptide means that IL-1α and β remain in the cytosol soon after translation and indeed IL-1α remains cytosolic, not usually being found in the circulation or in inflammatory body fluids except during severe disease, which may be as a result of release from dying cells (120) or as a consequence of calpain-mediated cleavage of membrane bound IL-1α (121). IL-1α may in fact act as an autocrine, intracellular messenger in some cells.

After synthesis, proIL-1β remains primarily cytosolic until it is cleaved and transported from the cell. In mononuclear phagocytes, some of the proIL-1β is secreted from intact cells in a manner distinct from the secretion of the mature form of the peptide. ProIL-1β requires cleavage by an intracellular cysteine protease that is highly specific for cleaving at the aspartic acid-alanine (amino acids 116-117) position (122). This enzyme, known as interleukin-1 β converting enzyme (ICE) is constitutively expressed as an inactive precursor requiring two cleavages itself for enzymatic activity. The cleavage of proIL-1α appears to be carried out by several enzymes that are less specific than ICE. The IL-1β pro-piece (amino acids 1-116) is myristoylated on lysine residues, as is proIL-1α (123), but, unlike IL-1α, proIL-1β has no membrane form and proIL-1β is only marginally active (124).

1.3.1.2: Interleukin 1 Converting Enzyme (ICE)

Apart from the specific role of ICE in cleaving the mature form of IL-1β from pro-IL-1β a great deal of evidence is emerging for more general roles of ICE in general physiology and pathology. ICE is also known as Caspase 1 and is thought to play a role in the activation of a cascade of intracellular proteases leading to programmed cell death (apoptosis). At least ten members of the caspase family have been identified to date (125). Each is capable not only of degrading key cytostructural and reparative proteins but also of activating other caspase family members by cleaving the pro forms of the enzymes at aspartic acid residues. Caspase 1 was identified on the basis of its sequence homology to the pro-apoptotic *Caenorhabditis elegans* gene product, ced-3 (126). In the nematode *Caenorhabditis elegans,* genetically determined cell death has an essential requirement for the ced-3 gene (127). Consequently, caspase-1 (ICE) may have divergent effects on cell survival, depending upon which of its substrates is preferentially processed.

1.3.1.3: Physiological Regulation of IL-1 Activity

Structurally, the two agonists, IL-1α and IL-1β are very similar, being comprised of 12-14 β sheets in a barrel conformation (128,129). However, when examining regulation of gene expression, mRNA stability, translation, processing and secretion the two proteins are considerably different. Once released from cells, mature IL-1β is regulated by two antagonistic molecules: the soluble form of the type II receptor, which binds IL-1β with high affinity, and sIL-1ra, which competes with IL-1β for occupancy of cell surface expressed receptors.

1.3.1.4: IL-1ra

IL-1ra is the first described naturally occurring specific receptor antagonist of any cytokine or hormone-like molecule. Several groups were involved in the discovery of IL-1ra with Arend *et al.* describing IgG driven IL-1ra production by human monocytes in 1985. A cDNA for a secreted form of IL-1ra (sIL-1ra) was cloned from a human monocyte library (130) and synthesised as a 177 amino acid protein, with cleavage of a 25 amino acid leader sequence prior to secretion as a variably glycosylated 152 amino acid protein. A second cDNA for an intracellular form of IL-

1ra (icIL-1ra) was subsequently cloned and expressed as an 18 kDa, 159 amino acid protein (105). sIL-1ra protein is produced by virtually any cell that is capable of synthesising IL-1, with the possible exception of endothelial cells and hepatocytes. icIL-1ra is found constitutively expressed in keratinocytes and other epithelial cells but is also a delayed product of stimulated monocytes and macrophages (131,132).

A smaller isoform of IL-1ra was recently described by Malyak et al. as a major cytoplasmic constituent of LPS-stimulated human neutrophils (133). This 16 kDa protein was also detected in monocytes and the human hepatoma cell line HepG2 (134). This isoform bound less well to IL-1 receptors in comparison to the original two isoforms of IL-1ra (135) and the origin of this low molecular weight isoform of IL-1ra appears to be by alternative translational initiation. Thus, IL-1ra appears to be a family of proteins and is similar to fibroblast growth factor in possessing both secreted and cytoplasmic forms.

Elevated blood levels of IL-1ra have been described in patients with juvenile chronic arthritis (136,137), polymyositis (138), systemic lupus erythematosus (139-141) and RA (141,142). RA patients exhibited a lower ratio of IL-1ra to IL-1β in plasma both at baseline and following surgery in comparison to patients with OA or osteomyelitis (142). This observation suggests that IL-1ra production may be relatively deficient or inadequate in RA patients. Furthermore, IL-1ra production was enhanced, and the ratio of IL-1β to IL-1ra was decreased, in peripheral blood monocytes form patients with RA after clinical response to treatment with methotrexate (143,144) or gold injections (145).

In rheumatoid synovial fluid IL-1ra levels have been shown to be elevated (146-148), although soluble type I IL-1 receptors in these fluids may have obscured accurate measurement of IL-1ra by ELISA (146). The major source of IL-1ra in synovial fluid is the neutrophil (147), even though these cells produced relatively less IL-1ra and more IL-1β in comparison to peripheral blood neutrophils (148). In patients with acute arthritis speed of recovery has been correlated with the balance between IL-1ra and IL-1β levels in synovial fluid (91). In the case of chronic RA, although levels of IL-1ra are elevated in the synovial fluid and tissues this may not be sufficient to overcome the locally produced IL-1β.

The gene for the human secreted form of IL-1ra contains three LPS-responsive promoter elements, one of which was identified as an NF–κB binding site (149). A second of these three sites has been recently identified to bind PU.1 (34). However, in contrast to the role of PU.1 in the regulation of many other myeloid genes, the binding of PU.1 to the sIL-1ra promoter does not significantly regulate basal activity in macrophages. The binding of PU.1 does, however, appear to be essential for LPS induced IL-1ra secretion (34) and also for LPS induced IL-1β (150). Interestingly, a second PU.1 binding site has been identified which overlapped with the NF-κB site and is also a recognition sequence for the transcription factor GABP (34). The crystal structures of the three transcription factors, NF-κB, PU.1 and GABP suggest that they would not bind together and would in fact compete for the same site (151-154).

1.3.1.5: Biological Effects of IL-1

The biological effects of IL-1 are very diverse but generally involve the induction and suppression of various genes. For instance, IL-1 induces a variety of non-structural, function-associated genes that are characteristically expressed during inflammation, particularly other cytokines such as IL-6 and the entire chemokine family. The constitutive expression of housekeeping genes such as albumin, lipoprotein lipases and cytochromes, however, is somewhat suppressed. These divergent effects on gene expression may represent an attempt by IL-1 to increase the efficiency of the host's immune system in dealing with attack. In disease however, excessive IL-1 induction of genes such as inducible nitric oxide synthase, soluble type 2 phospholipase A2 and type-2-cyclooxygenase lead to chronic inflammation. Potent induction of MMP synthesis and inhibition of TIMP synthesis contributes to local tissue damage in the inflammatory tissues.

Levels of IL-1β in the circulation of animals or humans correlate with the severity of some disease states (155). Although this does not necessarily implicate IL-1 as being causative in the pathogenesis of the disease, there is convincing data to suggest this in some cases. IL-1ra, sIL-1RII, neutralising anti-IL-1 antibodies, antibodies to the IL-1RI and antisense oligonucleotides to IL-1RI have been used to show an important contributory role for IL-1 in several disease states reviewed in (156).

Bone is one of the most sensitive tissues to the actions of IL-1 (157). It regulates not only bone formation but also bone resorption (158) and is believed to have an important role in the pathogenesis of bone lesions associated with RA (159) and osteoporosis (160). Bone resorption is regulated by at least three events; osteoid degradation mediated through collagenase produced by osteoblasts (161), recruitment of new osteoclasts from their progenitors and activation of pre-formed and newly developed osteoclasts (162). IL-1 exhibits potent bone-resorbing activity by stimulating these three steps independently. IL-1 induces osteoclast formation by a mechanism involving PGs and, as in the case of TNFα, direct interaction between osteoclast progenitors and osteoblastic cells is required in the osteoclast recruitment induced by IL-1 (163). IL-1 also has potent bone resorbing activity, which can be attributed to its role as a stimulator of PGE_2, as demonstrated by partial inhibition of in vitro bone resorption in the presence of indomethacin. IL-1 can also act in synergy with other cytokines with bone resorbing activities such as TNFα and TNFβ. However, even with optimal synergy, IL-1 is considerably more potent than these cytokines in inducing bone resorption (164-166).

1.3.2: TNFα

Tumour necrosis factor α is a 17kDa polypeptide that exists in multimers of three identical subunits that contain several potential glycosylation sites. TNFα is a potent inducer of IL-1 and it often has synergistic effects with those of IL-1. On the endothelium TNFα induces prostacyclin release, expression of adhesion molecules and synthesis of other cytokines. TNFα also induces expression of adhesion molecules on, and is chemotactic for, neutrophils and macrophages; activates fibroblasts, osteoclasts and chondrocytes; induces fever and release of acute phase proteins. Increased efficiency of antigen presentation may also result from TNFα induced expression of HLA-DR antigens and increased IL-2R expression on T lymphocytes. In RA TNFα activates synovial fibroblasts, inducing increased collagenase and prostaglandin release and osteoclasts, resulting in increased bone resorption (167).

It has been proposed that TNFα plays a pivotal role in the pathogenesis of RA. In RA TNFα has been shown to be critical in driving the production of IL-1 in synovial

membrane cultures (168). Using TNFα neutralising antibodies Brennan and colleagues have shown that within three days IL-1 bioactivity had virtually disappeared. TNFα has also been shown to be a critical regulator of GM-CSF (59), IL-6 and IL-8 (169). The dominance of TNFα in the development of pro-inflammatory cytokine profiles in RA was first demonstrated using dissociated rheumatoid cell cultures (52,53,170). These findings were complemented by animal model data that showed TNFα to be critical in the development of arthritic pathology (171,172) and then finally proven with clinical data on the efficacy of anti-TNFα antibody (173-176) and TNF receptor-Ig fusion proteins (177).

Transcription of the TNFα gene is complexly regulated and the promoter region contains binding sites for multiple transcription factors including NF-κB, AP-1, NF-IL6 and NF-AT (178-180). The p38 MAPK signalling pathway has also been shown to play a role in controlling translation via possible effects on the 3' untranslated region (181). In the murine macrophage cell line, RAW 264, NF-κB was shown to be essential for induction of TNFα production (178,182) but in human lymphoid cell lines NF-AT rather than NF-κB was required for TNFα production (179,180). Recent work published by Foxwell et al. (1998), using adenoviral infection with IκBα, has shown that LPS induced TNFα production in human monocytes is NF-κB dependent (183).

In general, the majority of biological functions that can be attributed to TNF are shared by IL-1. Indeed, IL-1 and TNF often act in concert with one another, synergistically amplifying their solitary effects. For the purposes of this report the actions of TNF have not been specifically outlined in great detail, because of the similarities with IL-1 actions and because of the fact that the main focus of this thesis is on the balance between IL-1β and IL-1ra production.

1.3.3: Interleukin-6 and Oncostatin M

The biology of IL-6 has been reviewed in detail recently by Toshio Hirano (184). Consequently, what is given here is an overview of those aspects of IL-6 biology that are relevant to its role in health and disease. For the sake of this thesis, IL-6 was

mainly considered due to the fact that its production by PBMCs may be differently regulated compared to that of IL-1 and TNF.

IL-6 is a typical example of a pleiotropic cytokine that acts on various cells: IL-6 induces the differentiation of B cells to antibody producing plasma cells, T-cell growth and differentiation, the differentiation of myeloid leukemic cell lines into macrophages, megakaryocyte maturation, the neural differentiation of PC12 cells, the development of osteoclasts, and acute-phase protein synthesis in hepatocytes. IL-6 acts as a growth factor for myeloma/plasmacytoma, keratinocytes, mesangial cells, renal cell carcinoma, and Kaposi's sarcoma, and promotes the growth of hematopoietic stem cells. In addition, IL-6 inhibits the growth of myeloid leukemic cell lines and certain carcinoma cell lines (185-187).

Human IL-6 is a variably glycosylated, 22-27kDa secreted glycoprotein that serves as a prototype for a family of molecules including leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotropic factor (CNTF), cardiotrophin-1 (CT-1) and IL-11. These cytokines constitute the IL-6 related cytokine subfamily because of their functional redundancy, structural similarity, and sharing of a receptor subunit. OSM was identified by its ability to inhibit the growth of human melanoma cell line A375 (188,189). OSM is also a potent growth factor for AIDS-related Kaposi's sarcoma (AIDS–KS) (190,191) and enhances the secretion of IL-6 by AIDS–Ks cells. The functional redundancy that occurs among these cytokines is very broad. In particular, IL-6, LIF, OSM, and CT-1 induce macrophage differentiation in a myeloid leukemic cell line, M1 (187,192,193). IL-6, IL-11, LIF, and OSM all induce the growth of myeloma cells (194,195). IL-6, LIF, and IL-11 enhance IL-3-dependent colony formation of primitive blast colony-forming cells (187,196,197). IL-6, LIF, IL-11, and OSM stimulate the biosynthesis of acute-phase proteins in hepatocytes (46,186,198-200). IL-6, LIF, OSM, IL-11, CNTF, and CT-1 all act on the nervous system (201). The functional redundancy observed among the IL-6-related cytokine subfamily is largely explained by the sharing of the receptor subunit, gp130.

Of the IL-6-type cytokines, IL-6 is best characterised as being involved in the acute-phase response in RA. IL-6 is produced mainly by fibroblast-like cells in the synovium (22,202) and is found at high levels in synovial effusions (55,97,203). Levels of circulating IL-6 in the blood have been shown to correlate with serum

levels of CRP and other APP in RA patients (55). Recent studies have shown that IL-6, IL-11, LIF and OSM are all produced in large amounts at the site of disease activity, but IL-6 derived from synovial fibroblasts may be the major hormone-like mediator that induces the hepatic synthesis of acute-phase proteins in RA (204).

1.4: Regulation of Monocyte Activation

The balance between the production of pro- and anti-inflammatory cytokines is critical to the outcome of immune responses. Factors that regulate monocyte activation may well determine the outcome of an inflammatory response and define the perpetuation of inflammatory diseases such as RA. Although inappropriate activation of mononuclear phagocytes is not necessarily causative in the initiation of autoimmune responses, it is at least highly likely to contribute to the progression and severity of disease (42). Thus, this section will discuss factors that are important in regulating monocyte activation in RA. Monocytes play a central role in both the innate and adaptive inflammatory responses and thus the areas considered here will be loosely separated into immunologically non-specific activation mechanisms, such as LPS and biochemical modulation, and those that may be involved in the specific immune response, such as stimulation with immune complexes and T cells. Of the multitude of possible innate activation mechanisms that can drive monocyte/macrophage functions particular emphasis will be given to bacterial LPS stimulation as this is a classical stimulator of IL-1 secretion. In addition, biochemical modulation will be considered as an innate, possibly environment-dependent stimulator of monocyte function.

Of particular interest to our group has been the exploration of Na^+/K^+ATPase expression in rheumatoid mononuclear cells, how this may vary with disease and how this may contribute to monocyte activation. Consequently, the biochemical modulation of mononuclear cells used in this thesis is focused on manipulations of the Na^+/K^+ATPase and hence the effects of downstream alterations in intracellular cation concentrations.

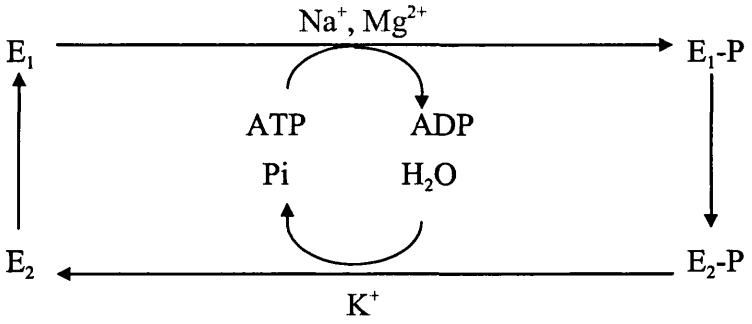
Immunologically specific modulation of monocyte/macrophage function involves interactions with antibody-coated pathogens or immune complexes, and intercellular interactions with other cells of the immune response, such as lymphocytes. Consequently, as immune complex and T cell interactions with monocytes might

play an important role in RA, IgG stimulation of monocytes through FcR and T cell-derived soluble mediator and membrane bound interactions with monocytes are considered here.

1.4.1: Innate Regulation of Monocyte Activation

1.4.1.1: Biochemical Modulation of Monocytes

The main biochemical manipulation used in these studies to regulate monocyte activation is the modulation of the Na^+/K^+-ATPase. The Na^+/K^+-ATPase (EC 3.6.1.3) is an integral heterodimeric membrane protein found in the cells of all higher eukaryotes and is responsible for translocating sodium and potassium across the cell membrane utilising ATP as an energy source. The enzyme was first described by Jens Skou in 1957 but it was not until 1966 that Oleg Jardetzky (205) proposed a model for the activity of ATPase.



The Na^+/K^+-ATPase can operate at a rate of 100 molecules of ATP per minute, each cycle expelling $3Na^+$ ions and importing $2K^+$ ions. In the model, shown diagrammatically above, the enzyme (E1) binds $3Na^+$ ions and one ATP molecule on its intracellular surface. E1 is phosphorylated on an aspartate residue (Asp 369), when both sodium and magnesium are present, which allows a conformational change in the protein structure resulting in a flip that exposes sodium to the outside of the cell. In this form the enzyme has increased affinity for K^+ ions. Two K^+ ions bind to the extracellular surface and upon dephosphorylation become internalised. As the conformation changes back to E1 it liberates the K^+ and is ready to start the cycle again.

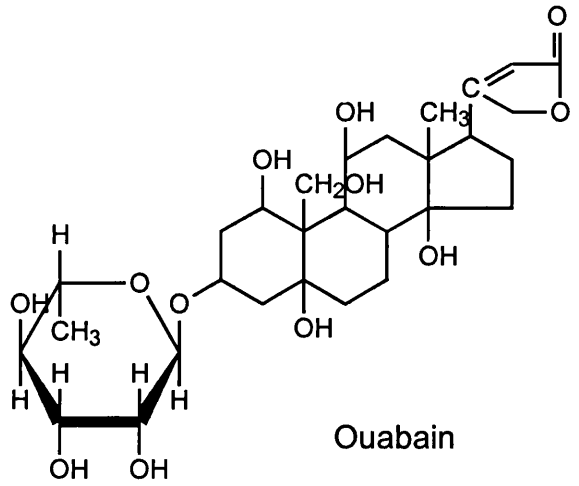
The Na^+/K^+-ATPase is composed of two subunits, an α unit of approximately 113kDa molecular mass and a β unit of approximately 55kDa molecular mass. The α subunit contains the ATP binding site, the phosphorylation site, and amino acids essential for the binding of cations and cardiac glycosides, which suggests that this subunit plays a major role in the catalytic function of the enzyme. The β subunit appears to be involved in maturation of the enzyme, localisation of the ATPase to the plasma membrane, and stabilisation of a K^+-bound intermediate form of the protein (206-209).

The ion gradients formed by this enzyme are essential for the Na^+-coupled transport of various nutrients, including glucose and amino acids, into cells (210), movement of such ions as Ca^{2+} (211,212) and H^+ (213) across the membrane, and osmotic balance and cell-volume regulation (214). Protein synthesis and receptor-mediated endocytosis and recycling also depend on the resulting ion gradients. These ion gradients also cause an electrochemical gradient which regulates the cellular membrane potential and thus plays a role in cell activity, muscle contraction and nerve activity (215). The importance of this enzyme cannot be overstated, and it has been estimated that approximately 25% of the ATP catabolised by an individual at rest is used by this enzyme.

Na^+/K^+-ATPase is the receptor for the cardiac glycosides, which were first described as therapeutic agents by William Withering in 1785 (216). Ouabain, as well as other members of the digitalis family, compete for the potassium binding site on the cell surface and inhibit the dephosphorylation of E2. Affinity labelling has shown that the site of interaction is primarily the α subunit but that some β subunit labelling also occurs (217). In the heart, ouabain has been shown to inhibit the Na^+/K^+-ATPase resulting in an increase in intracellular Na^+ and membrane depolarisation, which in turn results in an increase in intracellular Ca^{2+} via Na^+/Ca^{2+} exchange. The elevated calcium concentration subsequently causes an increase in the force of contraction.

Within the immune system ouabain has been shown to suppress T and B lymphocyte activation in vitro (218) and to induce IL-1β production by human monocytes (219). Such T cell hyporesponsiveness is indeed a feature of RA, where decreased release of T cell derived anti-inflammatory cytokines such as IL-4, IL-10 and IL-13 contribute to chronic inflammation (78,220-229) and a state of frustrated activation is seen

(230). Also in RA PBMCs show increased release of IL-1β /TNFα and decreased IL-6, whereas synovial fibroblasts show increased IL-6 with negligible IL-1 release. Recent studies by Matsumori et al. (231) have shown that various drugs used to treat heart failure, including the cardiac glycosides, modulate cytokine release from various cell types.



Ouabain

Research previously carried in our laboratory by A. Foey has centred upon the effects of ouabain in regulating the cytokine profile generated from healthy and rheumatoid inflammatory cells. In preliminary studies he showed that ouabain mimics the increase in IL-1β and TNFα production seen in rheumatoid monocytes (232) as does the calcium ionophore A23187 (233). The specific sodium ionophore monensin also stimulates the generation of a similar cytokine profile to ouabain in PBMCs (232) suggesting that ouabain may exert its effects initially via an increase in intracellular sodium. Evidence from Perregaux (1992) and later by Walev (1995) has also shown potassium regulatory effects on IL-1β processing in human monocytes. Using agents such as staphylococcal α-toxin and gramicidin, both of which selectively permeabilise plasma membranes for monovalent ions, the ionophores nigericin and valinomycin, and the Na^+/K^+ATPase inhibitor ouabain they demonstrated that K^+ depletion triggered processing of proIL-1β (234,235). The central role of K^+ depletion for inducing IL-1β maturation was demonstrated in cells permeabilised with α-toxin: processing of proIL-1β was totally blocked when cells were suspended in medium that contained high K^+, but could be induced by replacing extracellular K^+ with Na^+, $choline^+$ or sucrose. Walev et al. (1995) hypothesised that the activity of

ICE was linked directly or indirectly to potassium flux across the cell membrane or to local concentrations of potassium ions.

Elevation of intracellular Ca^{2+} could also play a role in the regulation of cytokine production through effects on gene transcription. The calcium/calmodulin-regulated protein phosphatase, calcineurin (PP2B), plays a critical role in the coupling of Ca^{2+} signals with cellular responses (236-238). Activated calcineurin regulates the activity of the nuclear transcription factor NF–κB via modulation of IκB degradation (239-242).

This compelling evidence, of ouabain induced pro-inflammatory cytokine induction and suppression of immuno-regulatory mechanisms, coupled with evidence of decreased Na^+/K^+ATPase activity in rheumatoid mononuclear cells demonstrated by K. Maubach and A. Foey (243) provided the impetus for the work presented here.

The decrease in rheumatoid mononuclear cell Na^+/K^+ATPase activity was assayed both functionally, with a histochemical assay, and by ^{3}H-ouabain binding studies. In the present studies, however, there was a need for a more quantitative ATPase assay and for a larger group of patients to be studied.

The functional consequences of decreased Na^+/K^+ATPase activity undoubtedly include altered processing of various cytokines, as shown with ouabain. However, the reason for this enzymatic abnormality is still an area for debate. One possible explanation for the impairment of Na^+/K^+ATPase activity in RA is oxidation and blockade of surface SH groups (243) by peroxides released during the inflammatory process. Studies in Jurkat T cells have shown that SH blockers induce membrane depolarisation and impair receptor mediated Ca^{2+} influx (244). Sulphydryl blockade with pHMPSA results in decreased ouabain binding ability.

Another explanation for the decreased Na^+/K^+-ATPase activity in rheumatoid mononuclear cells may lie in the discovery of an endogenous ouabain-like substance in mammals. The existence of a mammalian counterpart to the digitalis glycosides has been postulated for over a century (245). Hamlyn et al. reported in 1991, however, that one (perhaps the only) endogenous digitalis-like factor in human plasma was ouabain or a closely related isomer (246) secreted by the adrenal cortex (247). Whether the existence of this endogenous ouabain has any relevance to inflammatory diseases such as RA remains to be seen as most of the research so far

has centred upon cardiovascular conditions. Indeed, significant relationships between plasma ouabain concentration and cardiac index and mean arterial pressure imply that endogenous ouabain may be an important homeostatic factor in humans (248).

To date investigations into the role of intracellular cations in the regulation of monocyte cytokine secretion have not commented upon the balance between secretion of pro- and anti-inflammatory cytokines such as IL-1β and IL-1ra. Although there is now an expanding amount of literature on the role of cations, especially K^+, in the activation of ICE (234,249,250) and the processing of IL-1β at the time of these studies little was known about the effects of Na^+/K^+-ATPase modulation on the induction of monocyte secretion of IL-1β and IL-1ra.

1.4.1.2: Lipopolysaccharide (LPS) Stimulation of Mononuclear Cells

LPS of gram-negative bacteria induces a diverse array of biological responses in mammalian cells and initiates inflammatory, complement and coagulation cascades. The response of mononuclear phagocytes to LPS may be more relevant to reactive arthritis than RA but in this thesis LPS was mainly used due to its ability to stimulate IL-1 synthesis. Variations in responses to LPS between RA and normal monocytes were not the issue in question, rather the effects of LPS on other modulations of monocyte function, such as ouabain and T cell stimulation.

LPS has the ability to stimulate mononuclear phagocytes directly via cell surface receptors and indirectly via products of the complement cascade. By activating the alternative complement cascade LPS can initiate the innate inflammatory response via production of the opsonin C3b and the chemoattractant C5a. Mononuclear phagocytes express specific complement receptors, such as CR3, ligation of which facilitates phagocytosis of the C3b labelled organisms/tissue and release of lysosomal enzymes. The direct, most sensitive actions of LPS are mediated by CD14 and LPS binding protein (LBP). CD14 is a 55kDa glycoprotein that is found as a glycosylphosphatidylinositol (GPI)-anchored membrane protein (mCD14) on the surface of PMNs and monocytes and as a soluble protein (sCD14) in plasma. LBP is a 60kDa plasma protein that binds LPS (251). LBP has structural homology with plasma lipid transfer proteins and acts catalytically to accelerate the binding of LPS

to CD14 (252,253). While LBP was originally thought to play a direct role in inducing functional responses to LPS by acting as a coligand for mCD14, recent studies have shown that monomeric LPS presented as sCD14-LPS complexes is sufficient to trigger sensitive responses from endothelial cells (254,255), PMNs and monocytes (256), and the role of LBP appears limited to facilitating the translocation of LPS to CD14.

The precise role of CD14 in mediating LPS responses remains unclear, however, as it is thought to be unable to transduce signals across the plasma membrane (257-260). It is possible that CD14 plays a role in the internalisation of LPS from where LPS can then move into acidic intracellular compartments where the lipid A moiety may be partially degraded in ways that modify its biological activity (261-263). While some evidence suggests that LPS must be internalised before certain signals are transduced (264-266), other data argue that intracellularly processed LPS is not involved in signalling (267). Results presented by Vasselon et al. (1997) (268) suggest that one or more trypsin-sensitive cell surface proteins distinct from CD14 participate in both the uptake of LPS and the initiation of signalling (269). A member of the Toll-like receptor family (TLR4) has recently been suggested as a cell-surface co-receptor for CD14/LPS complexes and has been implicated in LPS stimulation of NF-κB activation (270).

Although definitive knowledge of the functional components of the LPS receptor is incomplete, it has been possible to analyse the intracellular signalling pathways induced by LPS-CD14 interactions (271). Weinstein et al first showed that LPS stimulated an increase in protein tyrosine phosphorylation in monocytes and identified MAP kinase (MAPK) isoforms as well as other proteins as targets (272,273). Using cells stably transfected with human CD14, LPS binding has been shown to induce rapid protein tyrosine phosphorylation of a 38kDa protein, termed p38, that is distinct from the 44- or 40kDa isoforms of MAPK (274). Other MAPKs activated by LPS-derived signals include the ERK family and JNK (275-277). The protein p38 is a kinase that is most closely related to the product of the HOG1 gene of *Saccharomyces cervisiae* (277,278). Both p38 and HOG1 are members of the MAPK family and define a new group of MAPKs with distinctive structural features (277). MAPKs require dual phosphorylation on threonine and tyrosine residues to achieve enzymatic activity. These phosphorylations are catalysed by dual specificity

protein kinases known as MAP kinase kinases or MEKs (279,280). LPS induced IL-1β and TNFα release from monocytes has been shown to involve p38 (181). p38 activation has been shown to be regulated by the small GTP-binding proteins Rac and Cdc42 and by p21-activated kinase-1 (Pak1) (281). MAPK-activated kinase-2 (MAPKAPK-2) is an in vivo substrate for p38, which in turn phosphorylates the small heat-shock protein Hsp27 (282,283). Other substrates for p38 include MAPKAPK-3 (284), MEF2C (285), Elk1 (286) and Max (287).

In addition to regulating the production of IL-1β and TNFα by monocytes, p38 also controls several other cellular responses. For example, p38 plays an essential role in IL-10 and prostaglandin H synthase-2 (PGHS-2) production by monocytes (288,289), and the production of PGHS-2, MMPs and IL-6 from fibroblasts and endothelial cells (290,291).

Signalling cascades initiated by LPS stimulation of monocytes have been shown to result in activation of several transcription factors, including NF–κB, NF-IL6 (C/EBPβ), AP-1, and members of the Ets family including Ets-2, Elk-1 and most recently PU.1 (292). In resting cells, NF–κB is complexed in the cytoplasm by an inhibitory protein, IκB. Signal-induced phosphorylation and subsequent proteolytic degradation of IκB frees NF-κB from such complexes. Following this, NF-κB rapidly translocates to the nucleus, binds to the κB element of target genes, and activates the expression of previously quiescent genes, such as IL-1, IL-6, TNFα, lymphotoxin and IFNγ. Many of the acute phase proteins also have κB sites in their promoters or enhancers and are produced as a result of NF–κB activation. These include serum amyloid A protein 1, α1 acidic glycoprotein, angiotensinogen, complement factor B and the C3 component of complement (293,294). Interestingly, anti-inflammatory drugs such as NSAIDs and dexamethasone inhibit NF–κB activation in cultured cells, suggesting that NF–κB may be a good target for potential therapies against chronic inflammatory diseases (295-298).

Quite how LPS initiates specific signalling in mononuclear phagocytes remains to be clarified. One thing that is clear, however, is that CD14 dependent pathways are involved in LPS induction of many pro-inflammatory functions. Of particular interest here is the ability of LPS to induce the production of pro-inflammatory cytokines,

such as IL-1β, TNFα and IL-6. LPS is also known to modulate IL-1ra production but to a lesser extent than IL-1β. In the case of IL-1β, LPS triggers transient transcription and steady state levels of IL-1β mRNA, which accumulates for four hours followed by a rapid decrease due to the synthesis of a transcriptional repressor (106,299). In contrast, stimulation with cAMP-inducing agents, such as PGE_2 or IL-1 itself, induces mRNA levels that are sustained for over twenty-four hours in human blood monocytes (300,301). Inflammatory effects of prostaglandins, such as PGE_2, may well involve regulation of IL-1 induced IL-1 release via modulation of cAMP levels. Interestingly, however, elevation of cAMP levels inhibits LPS induced IL-1β mRNA (302).

1.4.2: Immune Activation of Monocytes

As part of the adaptive immune response mononuclear phagocytes have the ability to interact specifically with immune complexes and lymphocytes. Immune complexes consisting of antibody and antigen interact with monocyte/macrophage Fc receptors facilitating inflammatory functions and phagocytosis. The interactions between mononuclear phagocytes and T lymphocytes are of a more complex nature. The classical scenario of antigen presentation to T cells is just one aspect of this interaction in which monocyte derived APCs regulate immune function. It is now known that T cells also regulate monocyte/macrophage functions via direct cell-cell interactions and via the secretion of immunomodulatory cytokines.

1.4.2.1: FcR Driven Monocyte Activation

Fc receptor biology has recently been reviewed extensively by Daeron (303). Consequently, what follows here is an overview of FcR functions that may be relevant to the regulation of monocyte activation.

Receptors involved in antigen (Ag) recognition include the B cell receptors (BCR), T cell receptors (TCR) and Fc receptors (FcR). FcR do not recognise Ag but the Fc portion of Ab's and can bind either Ab-Ag complexes or free Ab. Many FcR share activation motifs with BCR and TCR and may share similar signalling pathways.

FcR exist as membrane and soluble forms. Most human and murine FcR belong to the immunoglobulin superfamily (IgSF) with others belonging to the lectin family. FcR capable of triggering cell activation possess one or several intracytoplasmic activation motifs, which resemble BCR/TCR activation motifs (304). These motifs consist of twice repeated YxxL sequences flanking seven variable residues and are now designated as immunoreceptor tyrosine based activation motifs (ITAMs) (305).

Receptor aggregation, after ligand binding, is the first step in FcR signalling (306-308) and may involve the transphosphorylation of co-localised receptor chains (309). FcR aggregation may occur under two conditions, depending upon the affinity of the receptor for its ligand. High affinity receptors can bind monomeric Ig (non-complexed) with, for example, an affinity of $10^8 M^{-1}$ for macrophage FcγR (310). Low affinity FcR fail to bind monomeric Ig with a measurable affinity but do bind Ig aggregates or Ag-Ab complexes. All low affinity FcR belonging to the IgSF are IgG receptors. Interestingly, both low and high affinity receptors trigger cell responses with similar efficiency, the difference being in the number of events required for receptor aggregation. Monomeric antibodies bind to high-affinity FcR before forming complexes with multi-valent antigens, whereas antibodies are complexed to antigen before they bind to low-affinity FcR. By convention high affinity FcR are termed FcRI and low-affinity FcR are termed FcRII, with a subclass of the low affinity family being termed FcRIII.

Ligand binding FcRα subunits of multichain FcR are transmembrane molecules with 2 or 3 extracellular Ig-like domains of the V-type (311), rather than the C2-type as originally thought (312). They associate with a common FcRγ subunit (312) which has one ITAM motif in the intracellular domain. Association of the subunits is enabled via the interaction of a basic residue of the FcRα chain with an aspartic residue in the trans-membrane domain of FcRγ (313).

FcRγ are not members of the IgSF. They are related to TCRζ (314) and are encoded by related genes on chromosome 1 in both humans and mice (315). γ chains form γγ and ζζ homodimers, and sometimes γζ heterodimers, which can each associate with FcR and TcR.

FcRα genes are mainly expressed by myeloid cells and only FcγRIIIA are also expressed by sub-populations of lymphoid cells (312). FcγRI are expressed by

macrophages, monocytes and neutrophils. FcγRIIIA are expressed by macrophages, activated monocytes, NK cells, pre-B cells and T cells, notably of the γ/δ subset (316,317).

After aggregation of FcR it has been shown that signalling events occur within just a few seconds. In mast cells, FcεR1 aggregation has been shown to result in tyrosine phosphorylation of the FcRγ and FcRβ ITAMs within 15 seconds (318). TCRζ ITAMs are phosphorylated after aggregation of FcγRIIIA in NK cells (319) and FcRγ ITAMs are phosphorylated after aggregation of FcγRI in macrophage cell lines (320) and after FcαRI aggregation in U937 cells (321). Likewise, aggregation of FcγRIIA induces phosphorylation of their ITAMs in the monocytic cell line THP-1 (322,323).

Phosphorylation of ITAMs is correlated with the activation of several sets of cytoplasmic protein tyrosine kinases. The src family kinases have been shown to be activated by the aggregation of several FcR. FcγRIIIA activate lck in NK cells (324). FcγRI activate lck, lyn and hck in U937 cells treated with IFNγ (325). FcγRIIA activate lyn and hck in the monocytic cell line THP-1 (322). Lyn and hck also co-precipitate with FcγRIIA following its aggregation in THP-1 cells (322,323). Another set of kinases that become activated are the Syk family. Syk was found to be activated in human cultured monocytes triggered by FcγRIIIA (326), in THP-1 cells (327) and IFNγ-treated U937 cells (328) triggered via FcγRI and in THP-1 cells triggered via FcγRIIA (327). Syk was co-precipitated with FcRγ in macrophages following FcγRIIIA (326) of FcγRI (328) aggregation, and with FcγRIIA in THP-1 cells (329).

Tyrosine kinases phosphorylate various intracellular substrates including enzymes such as phospholipid kinases and phospholipases, adaptor molecules and proteins associated with the cytoskeleton. PI3-K is activated in various cell types, associated with the aggregation of various FcR including in U937 cells triggered via FcγRI or FcγRIIA (330). PLCγ-1 and PLCγ-2 have also been shown to be phosphorylated after the aggregation of various FcR in different cells including after FcγRI and FcγRIIA activation in THP-1 (327).

Signals induced by the aggregation of FcR with ITAMs join major biochemical pathways where they merge with signals generated by other receptors. These include

pathways leading to the increase in intracellular calcium, pathways leading to the activation of PKC and the ras pathway that results in the translocation of various transcription factors to the nucleus.

Once activated by syk, PLCγ generates metabolites that activate PKC and the inositide phosphate cycle. IP3 triggers the mobilisation of Ca^{2+} from the endoplasmic reticulum, which leads to a transient increase in intracellular calcium which in turn results in the opening of calcium channels in the plasma membrane and a sustained rise in Ca^{2+} concentration.

The FcγRIIIA has been shown to activate the ras pathway via the exchange factor sos (331), bound to the adaptor Grb2 that is recruited upon phosphorylation of shc. Ras phosphorylates raf, which in turn phosphorylates the MEK kinases which results in the phosphorylation of MAP kinase. MAP kinase activation has also been demonstrated after aggregation of FcγRI (325). MAP kinase activation leads to the activation of transcription factors such as NF-κB and NFAT.

The biological responses generated by FcR with ITAMs are cell type specific rather than receptor specific. FcγRI trigger super-oxide production and the secretion of inflammatory cytokines by macrophages and monocytes (332) via a mechanism that involves NF-κB. Also in human monocytes FcαRI induces the release of inflammatory cytokines (333), super-oxide, leukotrienes and prostaglandins.

Besides triggering cell activation, FcR with ITAMs internalise their cross-linking ligands. These can be either soluble immune complexes that are endocytosed in every cell type, or particulate immune complexes that are phagocytosed only by phagocytes. FcγRI and FcγRIIIA (334) as well as FcαRI (335) and FcεRI (336) mediate phagocytosis.

In summary, FcR ligation results in the activation of multiple signalling cascades. Through activation of PLCγ, PI3-K and ras signals from FcR have the capacity to modulate the translocation of multiple transcription factors. Which of these signalling cascades predominates during the activation of monocytes by IgG and how it regulates cytokine production is yet to be determined. The role of FcR aggregation in the interactions of immune complexes and monocytes in the rheumatoid synovium is also an area that has not yet considered the balance of pro- and anti-inflammatory cytokine production.

1.4.2.2: T cell Stimulation of Monocytes

Mononuclear phagocytes are capable of mediating tissue destruction via the direct secretion of MMPs (337) and indirectly by releasing cytokines such as IL-1β and TNFα which stimulate MMP release from connective tissue cells (338) and perpetuate inflammation. It is currently believed that chronic inflammation in diseases such as RA and MS is fostered by an imbalance between the production of pro-inflammatory cytokines and their inhibitors (339,340), e.g. IL-1ra production is deficient relative to IL-1β production in synovial tissue explants of patients with RA or OA (87,341). Although IL-1β and IL-1ra are produced by the same cell types (131), they are regulated differentially by a number of inducers. The critical events in the control of the balance between IL-1β and IL-1ra production have not yet been elucidated.

T lymphocytes are likely to play a central role in the pathogenesis of chronic inflammatory diseases such as RA. In the rheumatoid joint T cells that display a mature helper phenotype (i.e. CD3/CD4 positive) are the main infiltrating cell type in the pannus (342-344) and as they migrate through the synovium they become activated. Activated T cells bind to matrix proteins and fibroblasts in the perivascular space (345) and tissue damage occurs in areas where T cells are co-localised with monocytes. The ability of T cells to modulate monocyte activation has been an area of intense interest recently and will be discussed further here.

T lymphocytes are potent activating cells since they can release cytokines that stimulate B cells, monocyte-macrophages and fibroblasts (346). T cells regulate the inflammatory process by releasing cytokines or other soluble mediators that have pro- or anti-inflammatory properties. T helper cells are separated into Th1 and Th2 subsets based on their preferential production of IFNγ and lymphotoxin for Th1 cells and IL-4, IL-5, IL-10 and IL-13 for Th2 cells (347,348). IFNγ activates and primes macrophages to produce IL-1 in response to triggering events (349). In contrast, IL-4, IL-10 and IL-13 induce IL-1ra while down-regulating IL-1β production by macrophages (78,220-229).

Stout and Shuttles (1993) (350) demonstrated that T cells may trigger cytostatic and cytotoxic activity as well as the production of reactive nitrogen intermediates by macrophages (351) during direct cell-cell contact. Studies carried out by J-M Dayers'

group have complemented these data showing that direct cell-cell contact with stimulated T cells is a potent mechanism which induces the production of pro-inflammatory factors by monocyte-macrophages, including IL-1β, TNFα, IL-6 and MMP-1 and MMP-9 (352-354).

IL-1β secretion can be regulated by a variety of T cell derived cytokines, including IL-2, IL-4, IL-10, IFNγ and GM-CSF (78,355,356). However, little is known about the T cell-derived membrane signal which induces the production of IL-1β mRNA. The IL-1β mRNA inducing molecules are acquired by T cells rapidly after activation and are expressed independently of protein synthesis suggesting that the induction of monocyte IL-1 mRNA is mediated by a pre-existing T cell surface molecule that undergoes a change in either its expression and/or function following activation.

A number of T cell surface molecules are known to undergo rapid changes in function or expression following T cell activation, including CD11a, CD2, CD25, CD28, CD95 and CD69. Both CD11a and CD2 undergo a rapid and transient increase in binding affinity for their respective ligands, CD54 (ICAM-1) and CD58 (LFA-3), following engagement of the TCR (357-360). Although the role of such affinity changes in regulating the functions of ligand-expressing cells remains unknown, studies by Webb et al. (1990) have shown that the expression of IL-1β mRNA in human monocytes can be induced by either anti-CD58 or purified recombinant CD2 molecules immobilised to plastic (522). The CD40-CD40L interaction is also relevant to the contact-dependent induction of IL-1 and, in mice, nitric oxide synthesis by monocytes (362-364). The human lymphocytic cell line HUT-78, however, does not express CD40L (365) even though it is a potent stimulator of THP-1 cytokine production, as will be detailed later. Also, THP-1 cells do not express CD40. By interacting with CD11b-CD18 and CD11c-CD18, CD23 also regulates monocyte activation and production of IL-1β, IL-6 and TNFα (363). Similarly, anti-CD11a and anti-CD11b mAb partially inhibit IL-1β production induced upon contact with T cells (354,366). In addition, anti LFA-1 and anti-CD69 mAb have inhibitory activity (354). Previous work carried out by Landis et al. (1991) has also shown that the induction of IL-1β mRNA in monocytes during immune activation is regulated by direct contact with activated T cells (367). In later studies (368), anti-CD3 alone (in the absence of CD80/86) was used to stimulate T cells,

which then drove monocyte IL-1β mRNA in a CD2 dependent manner. Only anti-CD2 mAbs that inhibited the interaction between CD2 and CD58 (monocyte ligand) affected IL-1β mRNA production. As CD2 ligation by CD58 provides a co-stimulatory signal to T cells that promotes activation (369) to confirm that the anti-CD2 mAb was not simply reducing the activation of the T cells they were activated on plastic bound anti-CD3 prior to culture with the monocytes. The relevance of using T cells that had been activated by anti-CD3 alone to stimulate monocyte cytokine production is questionable, however, as various studies have shown that without co-stimulation through CD28 T cells do not display full activation characteristics (370).

In these studies McAllister et al. have also shown that anti-CD69 mAb significantly inhibited T cell induced IL-1β secretion by monocytes during anti-CD3 mitogenesis (52.1% inhibition). Anti-CD2 inhibited by 66% and combination inhibited by 83.8% (368). Interestingly, although blocking Abs against CD2 and CD69 inhibited T cell driven monocyte release of mature IL-1β protein the accumulation of IL-1β mRNA was not affected. Similarly, neutralising Abs against CD11a, CD18 and CD28 also had no effect upon production of IL-1β mRNA.

Upon activation, CD2 has been shown to undergo conversion to a form which binds CD58 with higher affinity (358,371). This conformational change has been suggested to allow triggering of monocytes through CD58 (372) and increased affinity for LFA-3 has been reported within fifteen minutes of T cell activation (371). However, in these studies agents that are reported to directly induce the change in T cell CD2 (i.e., forskolin and dibutyryl cyclic AMP) were unable to induce IL-1β mRNA production in monocytes. In fact, the ability of CD2 to undergo transformation is itself controversial.

CD69 is expressed within hours of T cell activation (373-375) and can be expressed independently of de novo synthesis via mobilisation of pre-existing intracellular molecules to the membrane (376). Previous reports (367) have shown that the induction of IL-1 secretion following contact mediated signalling was regulated largely by T cell cytokines. McAllister has shown, however, that membrane contacts are critical for the induction of IL-1β mRNA and that CD69 modulates the secretion. In addition mAb against CD69 have also been shown to prevent the induction of IL-

1β secretion in a number of monocyte cell lines when cultured with activated T cell lines (354,366).

The balance between TIMPs and MMPs has also been shown to be regulated by T cell cytokines and may be critical to the degree of tissue destruction in chronic inflammation (377). Shapiro (1990), Lacraz (1992) and Corcoran (1992) have observed that IFNγ, IL-4 and IL10 suppress MMP production whereas IL-6 and IL-10 increase TIMP expression (378). Through the release of soluble mediators and direct cell-cell contact with mononuclear phagocytes T cells may play a critical role in the regulation of extracellular matrix turnover.

Lacraz (1994) has presented evidence that a cell surface glycoprotein that is synthesised and presented on the surface membrane of stimulated T cells triggers the expression of MMPs in monocytes (379). Freshly isolated T cells as well as T cell lines induced, by direct contact, the production of two major MMPs, interstitial collagenase and 92kDa gelatinase. Fixed PMA/PHA treated T cells, J16 cells and Hut78 cells as well as membrane preparations from these cells induced production of the above mentioned MMPs in undifferentiated THP-1 cells. A marked synergistic response was also seen when a combination of LPS and activated T cells was used to stimulate THP-1 production of IL-1β and TNFα. PHA treatment of the T cells was insufficient to induce monocyte activation, negating the possibility of carryover and PMA carryover was also ruled out by substituting T cells with red blood cells. Using the THP-1 cell line Vey et al. have also shown that activated HUT-78 cell membranes induced IL-1β and IL-1ra production from THP-1 cells. IFNγ did not affect THP-1 response whereas differentiation with D3 increased IL-1β by 2.3-fold and IL-1ra by 1.6-fold (352).

Lacraz et al. (1994) challenged the ability of activated T cells to induce monocyte cytokine production using various methods. T cells treated with trypsin lost most of their capacity to induce 92–kDa gelatinase and IL-1β synthesis, whereas TIMP production was somewhat less affected (380). To test whether this protein was glycosylated, HUT 78 were treated with tunicamycin during activation. Tunicamycin treated Hut 78 cells were unable to induce THP-1 production of MMP, TIMP and IL-1β. Care should be taken when interpreting these data however due to the possible toxic effects of tunicamycin.

Peripheral T cells became fully competent to induce MMP release from THP-1 cells after 2 hours incubation with PMA/PHA. The ability to induce IL-1β was not maximally achieved until 48-72 hours culture, with initial responses seen at about 4 hours. Interestingly, J16 cells acquired the ability to induce MMP release from monocytes more rapidly than T cells (within 1hour). Also, induction of IL-1β release maximised after 1-4 hours of J16 activation and dropped with further J16 activation. The data suggest that the glycoprotein responsible for the induction of MMPs is expressed early and that the molecule responsible for cytokine induction is more variably expressed.

Lacraz et al. also found that T cell activated IL-1β release from THP-1 cells was suppressed by anti-CD11b and CD11C and CD69 (380). None of the these Abs inhibited MMP induction.

Activation of T cells may induce a conformational change in the membrane-associated protein. Alternatively modification of an existing membrane protein, e.g. by phosphorylation or proteolysis, may be required for activity. Also, activation may prompt the translocation of an already existing pool of proteins to the membrane. The persistent capacity of activated T cells to function on monocytes implies that de novo synthesis of the operative glycoprotein also occurs during prolonged T cell activation.

Conclusive evidence remains to be found as to which T cell membrane proteins and monocyte ligands are critical for the induction of contact-driven monocyte cytokine production. In spite of this confusion, however, recent evidence has been reported by Vey et al. on the signalling cascades that are activated within THP-1 cells upon stimulation with T cell membranes. Staurosporine, genistein, calyculin A, calphostin C, tryphostins, phenylarsine oxide, vanadate and okadaic acid (inhibitors of phosphorylation and dephosphorylation) were tested against HUT-78 membrane induced THP-1 cytokine production (381). Only okadaic acid, an inhibitor of serine/threonine phosphatases, dissociated IL-1ra and IL-1β production (Okadaic acid does not inhibit the PP2B-calcineurin). 50nM okadaic acid markedly inhibited secreted and cell associated IL-1ra, whereas secreted and cell associated IL-1β was increased. Okadaic acid did not induce IL-1β secretion from THP-1 cells in the absence of stimulation with HUT-78 membranes. This demonstrates that IL-1β and its inhibitor IL-1ra are inversely regulated by serine/threonine phosphatases upon

contact with stimulated T cells, IL-1β being down-regulated while IL-1ra is up-regulated.

Further results demonstrated that okadaic acid-sensitive serine/threonine phosphatases specifically modulate the production of IL-1β and IL-1ra by regulating the expression of their respective mRNA in THP-1 cells (381).

Little is known about the intracellular signals induced by direct cell to cell contact that regulate the production of the pro-inflammatory cytokine IL-1β, and its inhibitor IL-1ra. The identity of specific IL-1 inducing surface interactions is also questionable but probably involves both adhesion molecules and T cell activation molecules. In fact, antibody cross-linking of ICAM-1 (CD54) in a rheumatoid synovial cell line (E11) has recently been shown to induce activation of the transcription factor AP-1 and promote IL-1β gene transcription (382). Whether ICAM-1-induced signals can be replicated in normal monocytes remains to be seen, as does whether physiological ligation of ICAM-1 is sufficient for signal induction. The convergence of several signalling cascades may be necessary to initiate IL-1 induction following T cell-monocyte interaction. It has been shown that phosphatase inhibitors such as calyculin A (373) or cyclosporin A (383) decrease the production of pro-inflammatory cytokines by LPS-stimulated macrophages. In contrast, from recent studies it appears that okadaic acid potentiates PMA-induced IL-1β by increasing IL-1β mRNA and protein expression via effects on AP-1 binding (384,385).

In RA monocytes receive activating stimuli from interactions with T cell membranes, T cell-derived soluble factors, inflammatory mediators such as PGE_2 and PGI_2, and the oxidative environment. This fascinating milieu somehow converges in a signal that favours the production of pro-inflammatory cytokines from the monocyte/macrophages that encounter it. Whether T cell-derived stimulation of monocytes, which occurs in the rheumatoid synovium, can individually dominate the induction of pro- or anti-inflammatory cytokine secretion remains to be determined. Also, the level at which T cells can regulate monocyte cytokine production, whether it be increased transcription, translation or processing of cytokines remains to be determined.

RT-PCR analysis has shown that IL-1ra mRNA was present in unstimulated THP-1 cells which did not produce IL-1ra protein. Upon contact with activated HUT-78

cells, IL-1ra production was strongly induced, with no change in mRNA expression. Thus the modulation of IL-1ra production by membranes of stimulated HUT-78 cells may occur at a post-transcriptional level by stabilisation of mRNA, or at a translational level by derepression of IL-1ra mRNA translation. Both phenomena have been previously reported in different systems (108,386), but the mechanism involved in IL-1ra mRNA expression may be different since the 3' UTR of IL-1ra mRNA lacks the AUUUA sequence involved in the mRNA half-life and derepression processes (387).

IL-1β production depends on newly synthesised mRNA whose expression is inhibited by okadaic acid-sensitive serine/threonine phosphatases, and IL-1ra production depends on the derepression of pre-existing mRNA whose expression is triggered by dephosphorylation catalysed by serine/threonine phosphatases. The question as to whether similar phosphatases are responsible for both phenomena as well as the identity of these phosphatases and the nature of their substrates remain to be elucidated. However, such observations could imply that pharmacological intervention at the level of serine/threonine phosphatases might reverse the imbalance between pro-inflammatory IL-1β and its inhibitor IL-1ra which occurs in chronic destructive inflammatory diseases.

1.4.2.3: Differential Regulation of Monocyte Activation by Th1 and Th2 Cells

Vey et al. (1997) have shown that when monocytes were activated by factors secreted by stimulated T cells in the absence of cell contact, production of IL-1ra was 60 times that of IL-1β (381). Since the inhibition of IL-1β-mediated effects requires approximately 100 fold excess of IL-1ra over IL-1β (340) soluble factors from stimulated T lymphocytes may stimulate the generation of a monocyte cytokine profile in which the effects of IL-1β are regulated by IL-1ra. However, when stimulated T cells are in direct contact with monocytes, as is likely to be the case at a site of inflammation, the production of IL-1β overwhelmed the inhibitory concentration of IL-1ra. Among the soluble factors produced by T cells, Th2 cytokines such as GM-CSF, IL-4, IL-10 and IL-13 have been shown to favour IL-1ra production in several biological systems (224,225,388-390). Interestingly, Th1 but not Th2 lymphocytes are involved in mechanisms of chronic inflammatory diseases

such as RA and MS (391), whereas Th2 type cytokines display anti-inflammatory activities in these diseases (340).

Work performed using murine cells has provided evidence that upon contact with plasma membranes of antigen activated Th1 cells, macrophages rapidly acquire the capacity to produce TNFα and nitric oxide (351,392). The contact dependent activation of macrophages is partly due to membrane-associated TNFα, but other factors, such as CD40-CD40L interaction, are involved (392-395).

IL-1β mRNA but not IL-1β protein is produced upon contact of human monocytes with paraformaldehyde fixed T cells, unless soluble factors are added to the co-culture (367). Similarly, intact CD4+ T cells activated by purified protein derivative of Mycobacterium tuberculosis (PPD) induce IL-1β production by peripheral blood monocytes (396). Ag (PPD or TT) activated T cell membranes induced THP-1 IL-1β production. T cell activity peaked at six hrs and was Ag-concentration dependent. TT stimulation led to Th2 cell phenotype (IL-4 producers) whereas PPD stimulation led to Th1 phenotype (IFNγ producers).

Th1 clones strongly induced the production of IL-1β by THP-1 cells (4 out of 4 clones tested) with levels ranging between 484 and 806 pg/ml (IL-1ra 0.9-7.8ng/ml). Th2 clones showed less induction of IL-1β, with levels ranging from 21-114pg/ml (IL-1ra 7.0-49.6ng/ml) (397).

Overall, the ability to induce IL-1β production by THP-1 cells was directly correlated with the production of IFNγ by T cells ($p<0.001$), and was independent of their IL-4 production. Conversely, IL-1ra production was directly correlated with the production of IL-4 by T cells ($p=0.003$), but inversely with their production of IFNγ ($p=0.001$). No clear relationship between the T cells capacity to produce IL-10 and THP-1 responses was seen.

In these studies reported by Chizzolini, Ag was presented to T cells on autologous APCs, therefore anti-CD3 and anti-CD28 combination was also used to activate T cells. Again, the same correlation was seen with the T cell cytokine secreting capacity. Soluble cytokines were used to stimulate THP-1 cells. IFNγ had no effect (5-5000U/ml) whereas IL-4 gave low IL-1ra (but no IL-1β). When assayed T cell membranes were found to contain 0.37 ± 0.15ng/ml IL-4. When equivalent amounts

of membrane associated and soluble IL-4 were used to stimulate THP-1 cells, higher IL-1ra production was seen in the first group.

These results are in agreement and extend those of Landis, C.R. et al (1991) showing that activity exists predominantly within the CD4+ Th1 group.

Th2 activity may lie in membrane associated IL-4. Increased activity of membrane bound IL-4 could be due to changes in steric conformation or more likely synergy with other membrane molecules. Th1 cell induction of monocyte activation may also be due membrane-associated cytokines, in this case IFNγ, and recent evidence has been published to support the existence of membrane bound IFNγ. The expression of IFNγ has been shown to be detectable by enhanced fluorescence cytometry on activated T cells (398).

Of all the T cell surface antigens considered here as being relevant to activation of monocytes, no evidence supports a difference between expression levels on Th1 and Th2 cells. As the Th1 subset is the predominant population in the RA synovium (82) it would be interesting to observe whether rheumatoid T cells regulate the balance of monocyte IL-1β and IL-1ra production in a similar way to the T cell clones used by Chizzolini above. The recruitment of Th1 cells to sites of inflammation may well be as a result of differential chemokine receptor expression. Th1 cells show marked staining for CCR5 and CXCR3, and are only occasionally positive for CCR3 (399). It appears that CCR5 is highly expressed on Th1 cells and rarely present on Th2 cells, whereas CCR3 is found almost exclusively on Th1 cells. CXCR3 is highly expressed on both subsets (399,400). MIP-1β appears to be a selective ligand for CCR5 (401,402), eotaxin for CCR3 (403-405) and IP10 for CXCR3 (406).

Upon activation within the inflamed synovium Th1 cells may modulate monocyte cytokine production via a complex interaction of surface expressed cytokines, such as IFNγ, adhesion molecules and activation markers such as CD69. Which membrane interactions are critical to T cell stimulation of monocyte IL-1β and IL-1ra production remains to be seen. It is possible that T helper cell subset-specific surface molecules favour the induction of monocyte IL-1β or IL-1ra production. From the evidence presented here, however, it is most likely that the surface phenotype of T helper cells simply governs their recruitment to the rheumatoid joint and that once

they interact with resident mononuclear phagocytes the key interactions are those of adhesion molecules and surface bound cytokines. T cell induced monocyte cytokine production is dependent upon the state of T cell activation therefore activation induced surface markers may play a critical role.

1.4.3: Summary of Signalling Events Involved in IL-1 Gene Regulation

Thorough discussion of the signalling cascades involved in regulation of the IL-1 family genes is beyond the scope of this introduction. A diagram summarising the data presented in the previous sections, however, is shown in **figure 1**. Possible signalling cascades initiated by ouabain, LPS stimulated CD14 and FcγR ligation are shown in the upper part of the diagram. The contributions from T cell membrane interactions and secreted cytokines are shown in the bottom half of the diagram.

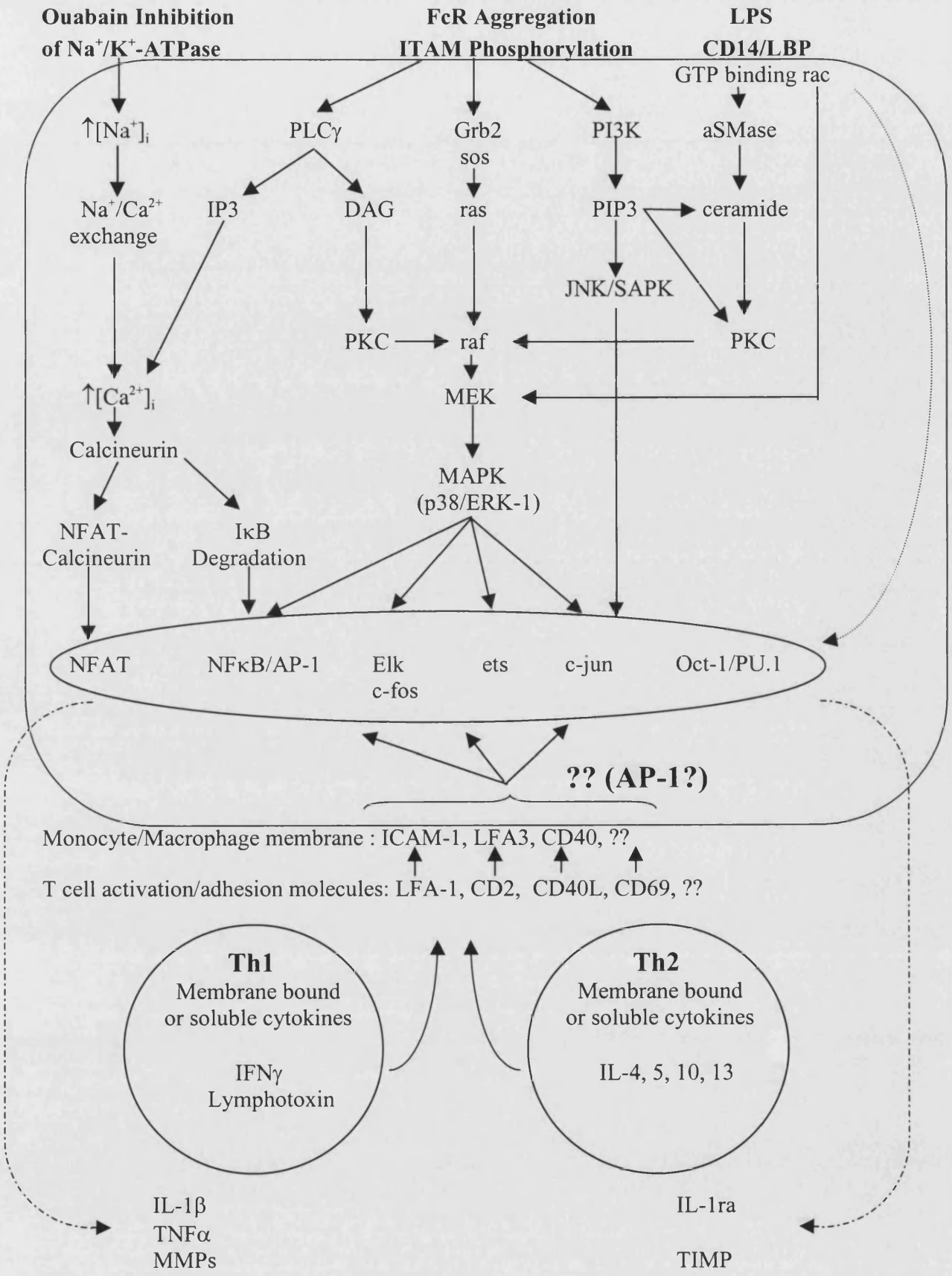


Figure 1: Summary of signalling molecules involved in regulation of IL-1 induction.

1.5: AIMS OF THIS STUDY

Despite intensive research efforts the factors that regulate monocyte cytokine production in rheumatoid arthritis remain unclear. Pro-inflammatory cytokines such as IL-1β have been identified as critical regulators of the pathogenesis of RA yet the conditions that govern their synthesis, secretion and biological activity remain somewhat elusive. Upon secretion the activity of IL-1β is controlled by the decoy type II IL-1 receptor and perhaps more importantly by the specific receptor antagonist protein IL-1ra. An understanding of the factors that determine the balance between IL-1β and IL-1ra production by monocytes would be greatly beneficial for the development of treatments for RA and indeed many other inflammatory conditions. Thus it was the primary aim of this thesis to identify features that differentially regulate the production of monocyte cytokines and in particular alter the balance between IL-1β and IL-1ra production.

One of the key membrane enzymes that has been shown to be involved in the regulation of monocyte cytokine secretion is the Na^+/K^+-ATPase. Inhibition of the Na^+/K^+-ATPase with ouabain in vitro has been shown to induce the production of pro-inflammatory cytokines by PBMCs via alterations in intracellular cation levels. In RA Na^+/K^+-ATPase activity has been shown to be reduced in erythrocyte membranes and early work from our laboratory has extended this to cells of the immune system, where decreased Na^+/K^+-ATPase in mononuclear cell membranes has been reported. Decreases in Na^+/K^+-ATPase activity in RA could be due to sulphydryl damage caused by the highly oxidative environment of the inflamed joint and this could well extend to effects on circulating cells.

Despite this strong evidence to support a role for the Na^+/K^+-ATPase in defining cytokine profiles in RA little work has been carried out to study the effects of Na^+/K^+-ATPase modulation under in vivo conditions. Hence one of the main aims of this thesis was to link the in vitro reports of ouabains pro-inflammatory effects with the reports of decreased Na^+/K^+-ATPase activity in RA by studying in vivo models of murine arthritis.

Upon the completion of the murine studies several questions arose as to the suitability of these models for assessing in vivo effects of ouabain. These studies did not provide definitive data for the pro-inflammatory effects of ouabain and thus

further studies were entered into, using human mononuclear cells, to improve the knowledge of the effects of ouabain on cytokine induction. Of particular interest was the study of the variation in sensitivity of rheumatoid and healthy PBMCs to ouabain induced cytokine production. By improving data on ouabain induced cytokine production and comparing effects of ouabain on rheumatoid PBMCs it was hoped to improve the current knowledge on how intracellular cation levels may contribute to the severity of the inflammatory condition in RA.

The study of ouabain induced production of pro-inflammatory cytokines was hoped to give valuable insight into the possible pro-arthritic role of decreased Na^+/K^+-ATPase activity in mononuclear cell membranes. A more pertinent line of investigation, however, was thought to be the study of the balance between pro- and anti-inflammatory cytokine production and in particular the balance between IL-1β and IL-1ra production. Consequently, one of the final aims of this study was to develop models for the investigation of IL-1β and IL-1ra production from cells of the monocyte/macrophage lineage. In the case of RA the activity of cells of the monocyte/macrophage lineage is regulated by various factors such as the oxidative environment, soluble mediators within the joint or peripheral circulation and contact with inflammatory tissues or other cells involved in the inflammatory response. Of particular interest was the role that T cells might play in regulating monocyte production of IL-1β and IL-1ra in the rheumatoid joint. Recent reports have demonstrated that helper T cells of the type I and type II phenotypes differentially stimulate the production of IL-1β and IL-1ra from monocytes that they interact with via the secretion of immune-modulatory cytokines such as IL-4, IL-10, IFNγ, MIF and IL-17, and also via direct cell-cell contact. Due to the reported enrichment of Th1 cells in the rheumatoid joint we were particularly interested in the reports that Th1 cells stimulate IL-1β production from monocytes via membrane specific interactions, whilst Th2 cells favour IL-1ra production. By developing models for the study of T cell driven monocyte cytokine production it was hoped that greater information could be gained as to how T cells regulate the balance between IL-1β and IL-1ra production by monocytes. Also it was hoped that T cells from various patient groups could be compared to healthy T cells for their ability to induce production of IL-1β and IL-1ra from the monocytic cell line THP-1. Secondly, it was hoped that monocytes from the different patient groups could also be compared to healthy

monocytes for their ability to respond to T cell signals derived from the J16 T cell line. By developing co-culture models we also hoped to be able to exploit them for the study of anti-inflammatory/rheumatic drugs. Whilst many studies have monitored the effects of potential therapies at modulating monocyte cytokine production in response to agents such as LPS we hoped to be able to demonstrate in vitro efficacy of compounds in co-culture experiments with conditions that are perhaps more relevant to the stimulation of monocytes in RA. Due to the award of a Basic Science Initiative grant from Pharmacia-Upjohn the specific goal of these later studies was to investigate the ability of Sulphasalazine to modulate T cell activation and the ability of T cells to stimulate monocyte production of Il-1β and IL-1ra.

Having developed the co-culture model it was possible to study the consequences of T cell and monocyte interactions in more detail. Of particular interest was the specific membrane interactions that are involved in T cell stimulation of monocyte cytokine production. At present the specific cell surface molecules that are critical for the transduction of signals from T cells to monocytes have not been defined. A great deal of evidence supports the involvement of adhesion molecules and activation markers such as CD69 but T helper cell sub-type specific antigens that regulate monocyte activation have not been identified. By developing a co-culture model for the study of T cell driven monocyte cytokine production the opportunity arose to further investigate the principal molecules involved in the membrane interactions. It was felt, however, that rather than using antibody blocking experiments a more relevant method for assessing the role of a surface protein in cell-cell interactions was to clone the molecule into a neutral cell type. In an attempt to address the role of T cell expressed CD69 in regulating monocyte production of IL-1β and IL-1ra experiments were carried out to clone and express human CD69 in COS-7 cells.

CHAPTER 2

MATERIALS AND METHODS

2.1: MATERIALS

All routine chemicals and reagents mentioned herein were purchased from Sigma Chemical Company Ltd., unless otherwise stated. The composition of buffers and growth media are detailed in Appendix 1. Tissue culture media concentrates and supplements were from Gibco BRL technologies, except the foetal calf serum that was from Sigma Chemical Company Ltd.

2.1.1: Antibodies

Several hybridomas, obtained from the American Type Culture Collection (ATCC) (Rockville, Maryland USA), are maintained by colleagues within the University and included CD2 (OKT11), CD3 (OKT3), CD25 (HB8784), CD69, CD14 (UCHM1) and HLA-DR4 (L243). The purified antibodies from these hybridomas were a kind gift from D. M. Sansom. The anti-CD19 antibody BU12 was a kind gift to D. M. Sansom from I. MacLennan (University of Birmingham, UK). The anti-CD80 (BB1) and the anti-CD28 antibody (9.3) were kind gifts to D. M. Sansom from P. Linsley (Bristol-Myers Squibb, Seattle, USA). Anti-mouse IgG ($F(ab)'_2$ specific) and anti-mouse polyvalent (anti-IgM, IgG and IgA) FITC was purchased from Sigma Chemical Company Ltd. Antibodies for ELISA of IL-1β, IL-1ra, IL-6 and TNFα were purchased from R&D Systems Ltd.

2.1.2: Molecular Biology Reagents

AquaPhenol was supplied by Appligene (Co. Durham, UK). Bacto-Agar, Bacto-yeast extract and Bacto-tryptone were obtained from Oxoid (Bristol, UK). Routine agarose from Sigma was used for resolving PCR products. DNA markers and restriction endonucleases, with appropriate digestion buffers, were supplied by New England Bioloabs (NEB).

2.1.3: Cell lines

The murine monocyte/macrophage cell line P388-D1 was a kind gift from Knoll Pharmaceuticals. The human monocytic cell line THP-1 was obtained from ECACC and the human T cell line Jurkat (J16) from the ATCC.

2.1.4: Transfected cells

CHO cells transfected with the CD80 receptor, as previously described (407), were a kind gift from D. M. Sansom. COS-7 cells (ATCC) that were transfected with CD69 cDNA were prepared as part of the studies in this thesis (see Chapter 7). Cells were assessed for surface protein expression using FACS analysis.

2.1.5: General equipment

Tissue culture plastics were purchased from Fahrenheit Laboratory Supplies Ltd, and cell culture was performed using sterile techniques in a laminar flow hood (Class II). All cell-based assays were incubated in a 5% CO_2 humidified incubator kept at 37°C. All PBMC and monocyte manipulations were carried out in polypropylene tubes to avoid loss through adherence. Where monocytes needed to be recovered after incubations, e.g. for FACS analysis, sterile polypropylene tubes were used (Falcon 6ml capped FACS tubes). Cells were counted using a Neubauer haemocytometer.

An Ultraspec II spectrophotometer (LKB Biochrom) was used to perform the ATPase assay as well as RNA and DNA quantification. Protein determinations (BioRad assay) and ELISA plate readings were carried out on a Dynatech MR5000 spectrophotometer. Proliferation assays were determined by a B Liquid Scintillation Top Counter (Packard).

FACS analysis was performed on a Becton Dickinson FACStar Plus, using a 100mW, 488nm argon laser with light being channelled by an FL-1 filter (520nm±20) and FL-2 filter (580nm±20).

2.2: GENERAL METHODS

2.2.1: Basic tissue culture

All cell lines were maintained in 75 ml culture flasks and incubated in a 5% CO_2 humidified incubator kept at 37°C. Specific media and supplement information is detailed in Appendix 1. Cultures were initiated from frozen stores held in Bath and at Knoll Pharmaceuticals or from growing cultures obtained from ECACC. Frozen vials of cells were rapidly defrosted in a water bath (37°C), the cell suspension then being washed twice in warm medium before culture. THP-1 and P388-D1 cells were maintained between $0.2x10^6$/ml and $1x10^6$/ml and J16s between $0.2x10^6$/ml and $2x10^6$/ml.

Chinese Hamster Ovary (CHO) cells and COS-7 cells were maintained in Dulbeccos Modified Eagle Medium (DMEM) supplemented as shown in Appendix 1. Both CHO and COS cells were passaged every 2-3 days after they had reached a state of confluence. To remove the adherent CHO/COS cells the medium was removed by aspiration and then the cells were washed once with phosphate buffered saline (PBS) before the addition of trypsin-EDTA (GibcoBRL technologies) (1ml for a $75cm^2$ flask). The cells were then incubated for five minutes at 37°C to allow the enzymatic digestion of the cell-plastic adhesion. Gentle tapping of the flask completely dislodged the cells after which fresh medium was added to inactivate the trypsin. At this point the cells were either taken for use or split approximately 1 in 10 in medium for continued culture. On occasion cells were taken at the sub-confluent stage for storage under liquid nitrogen.

2.2.2: Cell fixation

Prior to use, cells for membrane bound stimulations were fixed with 0.025% glutaraldehyde. This procedure leaves the cell intact allowing the surface molecules to be utilised, but negates the possibility of soluble mediator production or continued proliferation. To achieve fixation the cells were washed twice with PBS, counted and then resuspended for 2-3 minutes at room temperature with 0.025% glutaraldehyde (1ml for every 5 million cells). Fixation was terminated by the addition of medium

and the cells were washed twice in medium to prevent carry over of glutaraldehyde into cultures.

2.2.3: Freezing of cells

Cells for freezing were resuspended at $10x10^6$ per ml in 40% FCS culture-medium. An equal volume of 20% DMSO (in medium) was then added to the cell suspension in a cryovial and then frozen at -80°C for at least 10 hours prior to storage in liquid nitrogen. THP-1 cells were frozen using 10% glycerol as a preservative instead of DMSO due to the differentiating effects of DMSO on monocytic cell types.

2.2.4: Peripheral blood mononuclear cell (PBMC) preparation

Blood samples were obtained from healthy human volunteers or patients attending clinics at the Royal National Hospital for Rheumatic Diseases, collected in heparinised 50ml falcon tubes (100U/ml) and diluted 1:1 with PBS before being layered onto a lymphoprep gradient (Nycomed, 1.077g/ml density). The tubes were centrifuged at 350g for thirty minutes at room temperature and then the plasma/lymphoprep interface cell layer was collected. In some cases the plasma was retained for cytokine analysis. PBMCs were washed three times with RPMI 1640 medium and then counted.

2.2.5: Monocyte Preparation

Various methods were tested for the preparation of purified monocytes during these studies, including adherence selection, Percoll and Nycomed density gradient separation and magnetic-bead based immuno-magnetic selection. The quickest system that yielded the purest population of resting cells was that which utilised the Miltenyi Monocyte Negative selection kit (Miltenyi Biotec). The kit was used as per instructions, with a Variomacs magnet and LS+ columns and adapter. Briefly, PBMCs were washed in buffer (PBS supplemented with 10% autologous serum, 0.5% BSA and 2mM EDTA) then resuspended in fresh buffer to a total volume of 60µl per 10^7 cells. 20 µl of FcR Blocking Reagent and 20µl of Hapten-Antibody cocktail were added per 10^7 cells and incubated for 5 minutes at 6-12°C. The cells

were then washed twice in buffer and resuspended as before. Blocking reagent was added again as were 20µl of anti-hapten microbeads per 10^7 cells. After 15 minutes incubation at 6-12°C the cells were washed, resuspended in 500µl of buffer and loaded onto a buffer treated column. Negatively selected monocytes were washed through the column with buffer and retained. Purity was assessed by CD14 staining and routinely found to be greater than 90%.

2.2.6: T cell purification

$50x10^6$ PBMCs, as prepared above, were placed in a petri dish at a concentration of $5x10^6$/ml, and incubated for 1 hour at 37°C in complete RPMI-1640 medium. Non-adherent cells were then recovered, washed twice with medium and pelleted in an 8ml screw top tube. The cell pellet was then resuspended in 500µl of anti-DR4 antibody (L243), 500µl anti-CD14 antibody (UCHM1) and 100µl anti-CD19 (BU12) (all at 2µg/ml) and incubated for one hour at 4°C on a rotator. The cells were then washed and incubated for another hour at 4°C with 100µl sheep anti-mouse IgG dynal beads (diluted with 400µl RPMI-1640). Cells that had been bound by antibody could thus be removed via the use of a magnet. The recovered negatively selected cells were analysed by flow cytometry and found to be greater than 95% CD3 positive.

2.2.7: Preparation and maintenance of peripheral blood T cell blasts

For the preparation of T cell blasts, PBMCs purified as mentioned above were stimulated with staphylococcal enterotoxin A (SEA) (10ng/ml) which binds HLA-DR molecules on the surface of antigen presenting cells, such as monocytes, macrophages and B cells, within the PBMC population. The same cells also provided the co-stimulatory molecules that synergised with SEA to activate T cells. The antigen responsive blasts were cultured in RPMI 1640 medium at a density of 2-$4x10^6$/ml for 8-10 days with medium added every 2-3 days. PBMCs or purified T cells were also blasted with PHA or combinations of phorbol ester (PMA or PDBu) and Ionomycin.

2.2.8: Proliferation assays: Stimulation of resting T cells

Proliferation assays were performed in triplicate, in 96-well round bottom plates at a volume of 200µl per well. In each well, 100µl of T cells ($5x10^4$), 50µl of medium or fixed CHO or CHO-CD80 cells ($2x10^4$) and 50µl of medium containing other stimuli such as PHA, phorbol ester, ionomycin, A23187 or soluble CD3 (OKT3) antibody. CD3 cross-linking in culture was achieved with an equal concentration of anti-mouse IgG. When the effect of drug manipulations was tested the T cells were pre-treated for at least thirty minutes before stimulation and drug levels were maintained throughout culture.

The plates were incubated at 37°C for 72hr at which point 50µl of tritiated ^{3}H-thymidine (1mCi) was added per well. After an additional 18 hours incubation the plates were harvested on to replica 96 well fibre filter plates (Packard) using a Packard 96 well harvester. Radioactivity was measured via a β liquid scintillation counter (Top Count, Packard). The counted CPM represent the ^{3}H-Thymidine incorporated into the DNA of the cells, during cell division. It is therefore an indirect measure of proliferation.

2.2.9: Human IgG Preparation

Human IgG was used during this study as a monocyte stimulus. The IgG was prepared in-house using the following method. 100mls of blood was taken from a healthy volunteer (in two 50ml tubes) and left until clotting had taken place. The blood was then centrifuged at 1000g and the serum collected from the top layer. The serum was then dialysed over-night against a 0.01M phosphate buffer (pH7.3). The dialysed serum was then mixed with DEAE sepharose beads that had been equilibrated with the same phosphate buffer and left to incubate for 2hrs at 4°C. The IgG solution was then filtered off the beads and adjusted with 10X [PBS] to achieve physiological isotonicity. After sterile filtering the IgG solution was assayed for protein concentration using the Bio-Rad DC protein assay (see below).

2.9.10: Bio-Rad DC protein assay

Protein (IgG) concentration determinations were made using a Bio-Rad colourimetric assay. This assay is based upon the binding of Coomassie brilliant blue (G-250) to basic and aromatic amino acids of the protein. Briefly 5µl of each sample is mixed with 25µl of reagent A (supplemented with 20µl of solution S for every 1ml) and 200µl of reagent B. A standard curve was constructed using BSA (0-3mg/ml). After a 15 minute incubation at room temperature optical density is measured at 750nm.

2.3: ATPase ASSAY

In this assay ADP is utilised as follows to effect the oxidation of NADH:

$$ATP \xrightarrow[ATPase]{Mg^{2+},\ Na^{+},\ NH_4^{+}} ADP + Pi$$

$$Phosphoenolpyruvate + ADP \xrightarrow[Pyruvate\ Kinase]{} Pyruvate + ADP$$

$$Pyruvate + NADH \xrightarrow[Lactate\ Dehydrogenase]{} Lactate + NAD$$

The cells to be used were first washed in PBS, THP-1 cells being resuspended at a density of $1x10^6$/ml and peripheral blood mononuclear cells at $5x10^6$/ml in PBS. The cell suspension was then sonicated with 3-4 five second pulses of 8-10 Hz to lyse the cell membranes and then kept on ice at 4°C. At this point, ouabain was added to test samples and the lysate incubated on ice for 30 minutes. The assay cocktail (see Appendix 1) was also prepared at this time by adding 0.25 mM β-NADH and 1.2 units/ml of both lactate dehydrogenase and pyruvate kinase and incubating at 37°C for 30 minutes. The assay was initiated by adding 800µl of cocktail to 200µl of cell lysate and the absorbance of 340nm UV light was measured to determine a start point. Further absorbance readings were taken over time and the effect of ouabain on the oxidation of NADH monitored.

So that the activity of cell lysates can be correlated with specific ATPase activity and that the Na^+/K^+-ATPase component could be defined, a calibration curve was constructed using exogenously added ADP (0, 10, 20, 40, 80, 160mM) in the absence of cell lysate.

2.3.1: Preparation of Murine Cells for ATPase assay

Early attempts to harvest murine peripheral blood mononuclear cells were aborted due to poor separation on a 1.087 g/ml ficol gradient. Instead, the P388-D1 cell line was studied as were peritoneal macrophages. The following protocol was used for the preparation of peritoneal macrophages.

Mice were killed by CO_2 asphyxiation and cervical dislocation. The skin over the abdomen was then peeled back gently to reveal the peritoneal wall. The peritoneal cavity was then distended by the injection of 2ml of air and filled with 5ml of warm heparinised RPMI-1640. After gentle massage of the abdomen the media was extracted using a large bore needle. The murine cells were prepared for the ATPase assay as previously described above.

2.4: MURINE MODELS OF ARTHRITIS

For all murine studies female BALB/c mice (20-25g, aged 10-12 weeks) from Charles River were used. Before the animal models of arthritis could be set up a toxicology study for ouabain had to be carried out. Ouabain was made up in 0.9% saline and five groups of two mice were dosed via intra-peritoneal injection of 0.1 ml. Ouabain was studied at 0, 4.25, 8.5, 17,34 and 170μg per kilogram, the animals being dosed daily for eleven days. Ouabain had no toxic effects on the mice at these doses so a dose of 34 μg per kilogram was decided upon for initial studies. Later experiments used 170 μg per kilogram in an attempt to increase effects of the drug.

2.4.1: Antigen Induced Arthritis

Animals were sensitised by sub-cutaneous (s.c.) injections into the flank of 100μl of an emulsion comprising equal volumes of a 2mg/ml solution of m-BSA (Sigma) in

sterile 0.15M NaCl and FCA (Difco), to which was added M. *tuberculosis* such that the final concentration of *Mycobacterium* was 1.5mg/ml. At the same time as the flank injections the animals also received an intra-peritoneal (i.p.) injection of $2x10^9$ heat–killed *Bordatella pertussis* organisms (50µl). Omission of B. *pertussis* results in the development of a mild form of arthritis (408). In these studies there was a possibility that any effects of ouabain on the progression of the arthritis may be undetectable due to the gross severity of the inflammation. As a result a parallel group of animals were set up for antigen induced arthritis in the absence of *Bordatella pertussis* with the hope that a more mild arthritis might be a more sensitive model. The sensitisation injections were repeated seven days later and a further twelve days later i.p. injections of ouabain were commenced, control groups receiving physiological saline. Two days after the start of daily i.p. ouabain 10µl of m-BSA solution (10mg/ml in sterile 0.15 M NaCl) was injected into the joint of the left knee using a Hamilton syringe fitted with a 26 gauge/3/8 in. needle. To ensure that the antigen was injected into the joint space, the knee was flexed and mild pressure with the shaft of the needle over the anterior surface located the patella ligament. After swabbing with alcohol, the needle was inserted through the patellar ligament into the joint space. Intra-articular injections did not necessitate shaving of the knee or administration of a local anaesthetic.

2.4.1.1: Preparation of Tissue Samples

Animals were killed by CO_2 asphyxiation and both hind legs removed. The femur and tibia were cut midway along their length and the knee joints trimmed free of skin and musculature. The joints were then placed in perforated plastic holders and fixed in 10% formol saline for at least 48 hours followed by decalcification in 5% formic acid for 48 hours with constant agitation (the formic acid being replaced after 24 hours). The joints were then washed under running tap water for 2 hours followed by dehydration in graded alcohols before being embedded in paraffin wax. The fixed joints were sectioned in the sagittal plane at 5µm thickness, with several sections being taken to represent the entire joint. The slide mounted sections were stained for visualisation of cells, proteoglycans and collagen using hematoxylin/eosin, toluidine blue (or Alcian blue), and van Giesen's stain, respectively.

2.4.2: Zymosan induced arthritis

Mono-articular arthritis was induced in the left hind knee joint by the intra-articular injection of Zymosan A. The Zymosan was made up by grinding in a pestle and mortar at 18mg/ml in saline, a 10µl injection thus delivering 180µg to the joint. Two days prior to initiation of arthritis the ouabain groups started treatment with daily 0.1ml intra-peritoneal injections of ouabain (initial studies received 34µg/kg, later changing to 170µg/kg). Control mice received saline.

The rapid development of inflammation required that animals were removed from the experiment from as soon as 1 day after initiation. Time course studies for the development of proteoglycan (PG) synthesis defects showed that days 1-7 were the most critical times as after this point the inflammation was starting to resolve. As a result in the majority of studies patellae were harvested on days 1, 2, 3, 4 and 7.

2.4.3: Murine Patellae Proteoglycan Synthesis Assay

2.4.3.1: Tissue preparation

The mice were sacrificed by asphyxiation in a rising concentration of carbon dioxide followed by cervical dislocation. The skin was then peeled back from both knees to expose the joints. Cuts were made across the inferior ligaments with a scalpel and lifting with fine forceps allowed dissection parallel to the ligament. When the tissue could be lifted up sufficiently to reveal the patellae a final cut was made across the superior ligaments freeing the patellae, attached ligaments and surrounding tissue. At this point care had to be taken to leave as much ligament as possible to allow easy dissection of patellae in future steps. The patellae were placed into individual wells of a 96 well plate containing 0.2 ml of DMEM per well, to hold them until they could be trimmed further to remove inflammatory tissues.

Under low power microscopy (x20), excess tissue was removed from either side of the patellae leaving undamaged bone and cartilage with the attached ligaments. The patellae were then incubated for three hours, in a humidified incubator at 37°C in 95% air/5%CO_2, in 0.1 ml DMEM containing 1.85 MBq ^{35}S [Na_2SO_4] (Amersham, UK) to measure "*ex vivo*" levels of proteoglycan synthesis.

2.4.3.2: IGF-1 Induced Proteoglycan Synthesis

Recombinant human Insulin-like Growth Factor (IGF-1) (Calbiochem) was prepared by first dissolving 250μg stock in 400μl 10mM HCl (containing 1mg/ml BSA) and then making up to 1ml in phosphate buffered saline (containing 1mg/ml BSA). The effects of IGF-1 stimulation of PG synthesis in control and arthritic patellae were studied at concentrations of 0, 75, 150, 300 and 600ng/ml, the arthritic patellae being taken on day 1 after zymosan induced arthritis. The patellae were incubated in 0.2ml DMEM containing the IGF-1 for 24hrs in a humidified incubator at 37°C in 95% air/5%CO_2, with 1.85 MBq ^{35}S [SO_4] per ml being added for the last 3hrs.

2.4.3.3: Measurement of ^{35}S [SO_4] Incorporation

After incubation the labelled patellae were transferred to a 96 well plate containing 0.2% PBS + 1mg/ml Na_2SO_4 for 5 minutes to remove unbound ^{35}S [SO_4] label. Labelled glycosaminoglycans were then extracted from the patellae by a modification of the method described by de Vries et al. (409). Tissues were fixed in 0.2 ml 4% phosphate-buffered formalin containing 0.2% cetylpyridinium chloride (CPC) for one hour at room temperature followed by decalcification in 0.2 ml 5% formic acid for 24 hours at room temperature. The patellae were removed intact from the surrounding tissue and digested at 60°C for 24 hours in 0.1ml sodium acetate pH 6.0 containing 2mg/ml papain (Sigma), 0.2M sodium chloride, 50mM ethylenediamine tetraacetic acid (EDTA) and 10 mM L-cysteine hydrochloride. The plate was sealed in a humidified atmosphere to prevent evaporation.

The glycosaminoglycans were precipitated by incubating 0.15 ml of the digest with an equal volume of 0.2% CPC in an Eppendorf tube for 2 hours. Centrifugation at 10000g for 5 minutes in a micro-centrifuge pelleted the precipitate in the Eppendorf tubes. The pellets were washed once in 0.2 ml of 0.1% CPC, recentrifuged and then resuspended in 0.2 ml water. The degree of ^{35}S [SO_4] incorporation was then measured by transferring 0.1 ml of the precipitate to a 96 well "lumaplate", adding 0.2 ml microscint fluid and counting on a Packard Topcount.

2.5: T CELL DRIVEN MONOCYTE CYTOKINES

2.5.1: THP-1 differentiation

In order that THP-1 cells could be used as a model for monocyte/macrophages they first had to be differentiated with $1,25(OH)_2$ vitamin D3 (hereafter referred to as D3). In their resting state THP-1 cells express very low levels of CD14 and respond poorly to LPS stimulation. THP-1 were suspended at a density of $5x10^6$/ml and incubated for 48hrs with 10nM D3. Differentiation was measured as an increase in CD14 expression with populations routinely becoming approximately 70% CD14 positive (see **figure 6.1A**).

2.5.2: T cell/J16 Activation

Phorbol esters, ionomycin and CD3/CD28 ligation were tested for their ability to activate T cells. T cell activation was measured via tritiated thymidine incorporation, in proliferation assays (detailed above) and as changes in activation marker expression using FACS analysis (see below). The effect of activated T cells and J16 cells on monocyte cytokine production was compared to that of resting cells in co-culture assays. For these experiments T cells and J16 cells were suspended at $1x10^6$/ml in RPMI-1640 and stimulated for 18hrs at 37°C. Before co-culture the T cells were assessed for activation by measuring surface CD69 expression.

2.5.3:Co-culture

The effect of T cell interactions on the production of monocyte cytokines was assessed using normal T cells, J16 cells and THP-1 cells as well as some experiments with purified PBMCs and monocytes. All experiments were carried out in 96 well round bottom polystyrene plates (Falcon). Differentiated THP-1 cells (see **method 2.5.1**) were suspended at $1x10^6$/ml in RPMI-1640 and 100µl added to each well. PBMCs or monocytes were suspended at $2x10^6$/ml and 100µl added to each well. The monocytic cells, in some cases, had been pre-treated with drugs and incubated at 37°C prior to addition of T cells. 100µl of activated or resting T cells or J16 cells, suspended at $5x10^6$/ml, were then added to the culture. At the same time any other stimuli, such as LPS, were added to give a total volume of 220µl per well.

To assess the contribution of membrane signals from the T cells, in the absence of soluble factors, the T cells (or J16 cells) were fixed according to the protocol for CHO cells outlined above (see **method 2.2.2**). Each experiment was duplicated in two wells with controls receiving 100µl of RPMI-1640 instead of T cells. Experiments were incubated for varying time-courses at the end of which supernatants were harvested by gentle aspiration of each well after the plate had been centrifuged for 5 minutes at 350g. Supernatants were stored in a clean 96 well plate and assayed immediately for cytokine content. Remaining supernatants were stored in a sealed plate at –18°C.

2.6: ENZYME LINKED IMMUNOSORBANT ASSAY (ELISA)

NUNC maxisorb plates were coated overnight with 100µl per well of primary antibody in bicarbonate buffer pH9.6. Prior to testing, antibody concentrations for optimal ELISA performance were determined by running titrations of coating antibody with different concentrations of detecting antibody. TNFα, IL-1β and IL-6 coating antibodies were used at 4µg/ml and IL-1ra coating antibody was used at 10µg/ml. To eliminate non-specific plastic adherence the plate was then blocked for 90 minutes with 300µl per well of PBS containing 1% BSA and 5% sucrose. After this the plate was washed three times before the addition of samples. For each ELISA a six point standard curve was constructed using recombinant human protein. All standards and samples were assayed in duplicate, with the samples being diluted in RPMI-1640. The plates were then sealed and incubated for two hours at room temperature of overnight at 4°C. The plates were then washed three times before the addition of 100µl of biotinylated secondary antibody, which was incubated at room temperature for two hours. TNFα and IL-1β detection antibodies were used at 100ng/ml, IL-6 detection antibody was used at 25ng/ml and IL-1ra detection antibody was used at 50ng/ml. After washing, the plates were incubated with 100µl streptavidin conjugated horseradish peroxidase (1µg/ml) for 20 minutes. The plates were then washed four times before the addition of 100µl OPD substrate (Sigmafast P-9187). After suitable colour had developed the reaction was stopped with 50µl of 0.5M H_2SO_4 and absorbance values read at 490nm.

2.7: FACS ANALYSIS

Surface staining was performed by incubating $2x10^5$ cells for 30 minutes at 4°C with 50µl of primary antibody (1µg/ml). Cells were then washed with PBS and any primary antibody bound to the cells was detected using FITC-conjugated anti-mouse polyvalent Ig (Sigma) at 1:300 dilution (7µg/ml). To improve the level of non-specific staining of monocyte/macrophages a $F(ab)'_2$ specific FITC-conjugated anti-mouse Ig was used (Sigma F-2653). As a control the staining of cells treated with only the secondary antibody was also determined.

2.7.1: Annexin-FITC Assay

The annexin-FITC binding assay (Apoptest™, Boehringer Mannheim) relies upon the affinity of annexin-V for binding phosphatidylserine (PS), which is rapidly externalised during apoptosis (410). Briefly, after experimental treatment, $3x10^5$ cells were washed once in ice cold PBS and resuspended in 100µl HEPES buffer containing annexin-FITC (2µl neat per test) and the nuclear stain, propidium iodide (PI) (final concentration 2µg/ml). After a ten minute incubation at room temperature the cells were diluted 1:5 with HEPES buffer and analysed by FACS. Annexin-FITC binding was visualised as FL-1 channel fluorescence and PI staining as FL-2 channel fluorescence. Plotting FL-1 against FL-2 allowed the distinction of necrotic (FL-1 +ve, FL-2 +ve) and apoptotic (FL-1 +ve, FL-2 -ve) cells.

In certain experiments, to measure general cell death, PI incorporation was used as determine membrane integrity.

2.8: MOLECULAR BIOLOGY

2.8.1: CD69 mRNA Extraction

J16 cells readily express CD69 on their cell surfaces within one to two hours of activation with phorbol ester and calcium ionophore. For mRNA extraction, $10x10^6$ J16 cells were stimulated at a density of $2x10^6$ cells/ml with 5ng/ml PMA and 1μM ionomycin for 2 hours in a 24 well plate. To isolate mRNA from Jurkat cells for subsequent PCR reactions a Pharmacia QuickPrep® Micro mRNA purification kit was used. This kit allows the isolation of polyadenylated RNA from small numbers of eukaryotic cells, bypassing the need for intermediate total RNA purification. For all RNA work only specific, reserved reagents, tips and pipettes were used to limit the possibility of RNAse contamination. All solutions were made up with Diethylpyrocarbonate (DEPC) treated water.

Briefly, the cell contents are extracted into an extraction buffer that contains a high concentration of guanidinium thiocyanate (GTC), ensuring rapid inhibition of endogenous RNAses. The extraction cocktail is then diluted with elution buffer which reduces the concentration of GTC sufficiently to allow the formation of hydrogen bonds between the poly(A) tracts on the mRNA and the oligo(dT) attached to the cellulose slurry. Incubation of the elution cocktail with the cellulose slurry, followed by salt buffer washing captures the mRNA which can then be eluted from the spin-column with 65°C elution buffer.

2.8.2: mRNA Quantification

RNA quantification was carried out using the Ultraspec II spectrophotometer (LKB Biochrom) according to the instruments instructions. Briefly 5μl of the RNA sample was diluted to a final volume of 125μl and the optical density ratio between 260nm and 280nm was calculated. Typically the latter was between 1.8 and 2, indicating low levels of impurities. Concentration of RNA was calculated according to the equation:

$$\mathbf{A_{260} \times 40 \times dilution = [RNA]/(mg/ml)}$$

2.8.3: Reverse Transcribed Polymerase Chain Reaction (RT-PCR)

Siliconised plastics were used for these reactions in order to prevent the newly formed cDNA from sticking. The following reaction was set up containing approximately 400ng total RNA:

SuperScript RT enzyme (200U/ml) (Amersham)	2µl
RT buffer (5X) (Amersham)	6µl
DTT (0.1M) (Sigma)	3µl
dNTPs mix (10mM) (Pharmacia)	1.5µl
Oligo-dT (45µM) (Pharmacia)	1µl
RNAse inhibitor (Pharmacia)	1.5µl
DEPC water	to 30µl

The reaction mixture was incubated at 37°C for 1 hour and stopped with a 5 minutes incubation at 95°C. Samples were kept on ice when PCR reactions were to be performed immediately or alternatively stored at -20°C.

2.8.4: PCR primer design.

Primers were designed using Mac vector and produced by Gibco BRL custom primer service. See **section 7.3.2** for primer details.

The sequences of the primers utilised in this study (5' to 3') were

CD69 Forward: GGA ATC TTG AGA ATA AAG ATG AGC

CD69 Reverse: GTT TCC TTA TTA TTT GTA AGG

2.8.5: PCR conditions

PCR reactions were set up with 2µl of the freshly prepared cDNA together with 6µl of 25mM $MgCl_2$, 10µl of 2mM dNTPs, 1µl of each of the primers (stock of 1mM) and 10µl of Taq buffer (Promega). Water was added to a volume of 100µl and reactions were covered with mineral oil and performed with a Robocycler gradient

96 (Stratagene). The PCR cycle was initiated with a preincubation for 5 minutes at 95°C, to denature the coiled DNA, followed by the addition of 0.5µl of Taq enzyme (Promega). After that the following cycle was performed 30 times

-1 minute at 95°C (denaturing)

-1 minute at 54°C (annealing: - Temperature depends upon Tm of primers)

-1 minute at 72°C (extension)

A final extension incubation at 72°C was performed before chilling the reaction mixtures to 4°C.

2.8.6: Restriction Digests

Restriction digests were carried out to prepare plasmid DNA for ligation reactions and to map cloned DNA fragments. Restriction enzyme usage depended upon the particular requirements of individual experiments and details are included where necessary with the relevant results. Digestions were carried out in 20µl volumes unless larger amounts of DNA were required for gel excision for further manipulation. A typical restriction digestion reaction was compiled as follows:

Plasmid DNA (0.5µg/ml)	5µl
Enzyme specific buffer (10X)	2µl
Restriction Enzyme (3.5µg/ml)	1µl
Water	to 20µl

Restriction enzyme content was limited to a maximum of 10% of the reaction volume to prevent effects of contamination with the carrier glycerol.

Reactions were carried out on a heating block at the required temperature for the enzyme being used. Samples were electrophoresed on 1% (w/v) agarose gels for analysis on a UV transilluminator. Digested DNA was cleaned via phenol-chloroform extraction followed by precipitation with sodium acetate in ethanol. Briefly, the digested sample was expanded to 100µl with water. To this was added 100µl of phenol and 100µl of chloroform. The sample was then vortexed vigorously before being centrifuged briefly at 10,000g in a micro-centrifuge. The upper aqueous

layer was gently removed and kept and a repeat extraction performed on the remaining sample. The retained aqueous phase was then mixed with a one tenth volume of sodium acetate (3M) and two volumes of absolute ethanol. The mixture was then chilled at –18°C for five minutes to allow the formation of the DNA precipitate. The precipitate was pelleted by centrifugation and the alcohol removed. After the pellet had been air-dried it was resuspended in 20µl of water.

2.8.7: Ligation Reactions

DNA ligation was necessary for the generation of recombinant plasmid DNA, in the cloning of CD69. The following reaction was used:

Plasmid DNA (50ng/µl)	1µl
cDNA (50ng/µl)	1µl
Ligase buffer (10X) (New England Biolabs, NEB)	1µl
Ligase Enzyme (T4 DNA ligase 400,000U/ml) (NEB)	1µl
Water	6µl

Ligation reactions were carried out in Eppendorf tubes at 16°C for four to sixteen hours.

To achieve optimal ligation a vector: insert ratio of 1: 3 or 1: 5 molar ends of DNA was used. With information of the size of vector and size of insert available the amount of insert DNA needed for a given amount of plasmid DNA in the ligation reaction (50-100ng) was calculated as follows:

$$\text{ng of insert} = \frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \text{ X Molar Ratio of } \frac{\text{Insert}}{\text{Vector}}$$

Ligation reactions were terminated by heating to 70°C (to inactivate ligase enzyme) before DNA was either used to transform bacteria or stored at –20°C. Controls such as plasmid self ligation in the absence of insert were used to assess possible transformation efficiency of recombinant clones. Plasmid DNA was analysed on an agarose gel to determine linearisation.

2.8.8: Agarose gel Electrophoresis of PCR Product

Agarose gels were made in TAE buffer as a 1-2% solution (w/v), boiled in a microwave oven until the agarose was completely melted and then cooled to approximately 50°C. Ethidium bromide was added to a final concentration of 500μg/ml before the gel was poured into an electrophoresis tray. All DNA electrophoresis was carried out in TAE buffer (containing ethidium bromide) using a standard horizontal electrophoresis tank attached to a power source of 70 – 100 volts.

Gels were analysed over a UV transilluminator and photographed.

TAE buffer was made as a 50X stock as follows:

Trizma Base (Sigma)	242g
Glacial Acetic acid (Sigma)	57.1ml
EDTA (0.5M pH 8)	100ml
Milli Q water	to 1 litre

2.8.9: Transformation of Bacteria (Heat shock)

In order to identify recombinant clones of insert and vector DNA, TOP10F' (Invitrogen) bacteria were transformed via heat shock. For each transformation one 50μl vial of bacteria were used, to which was added 2μl of β-mercaptoethanol and 2μl of each ligation reaction. The cells were then mixed very gently and incubated on ice for thirty minutes. The bacteria were then heat-shocked at 42°C for exactly thirty seconds and then immediately placed back on ice for two minutes. At this point 250μl of SOC medium (at room temperature) was added to each tube and then the vials were shaken for one hour at 37°C. The transformed bacteria were then plated out on to LB agar plates (see below) containing 50μg/ml ampicillin. When all liquid was absorbed the plates were inverted and incubated at 37°C overnight.

2.8.9.1: Reagents required for bacterial manipulations

SOC medium (Invitrogen): 6ml vial containing 2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5mM KCl, 10mM $MgCl_2$ and 20mM glucose.

Luria-Bertani (LB) broth: 10g Bacto-tryptone (Oxoid), 5g Bacto-yeast extract (Oxoid) and 10g Sodium chloride made up to 1 litre with milli Q water and autoclaved.

Agar plates: 1% (w/v) Agar (Sigma) dissolve in LB broth then autoclaved before being cooled to 50°C for the addition of 50μg/ml ampicillin. Agar was then poured into petri dishes.

2.8.10: Selection of Transformed Colonies

Antibiotic treated plates selected colonies that had been transformed with plasmid-encoded antibiotic-resistance genes. To verify whether the individual colonies contained plasmid that contained the insert, PCR selection with CD69 primers was used. The DNA used for the PCR reactions was either prepared using a Wizard Miniprep system (Promega) or via the simple addition of a small dab of the bacterial colony into the PCR tube. In the later case cell lysis at the temperatures involved rendered the plasmid DNA available to the PCR reaction. Using 96-tube PCR plates it was possible to quickly screen many colonies for the presence of insert without the need of DNA preparation.

In some cases larger amounts of DNA were prepared for screening and analysis using the Miniprep system. Bacterial colonies were expanded overnight in 10ml of LB medium, supplemented with ampicillin (50μg/ml), contained in a 30ml tube and incubated at 37°C in a shaker. Aliquots of this culture were then used for DNA preparation as described in the kit. Briefly, 1.5ml of the overnight culture were pelleted by centrifugation at 6,500rpm in a bench-top micro-centrifuge for five minutes. The supernatant was aspirated and the pellet resuspended in 100μl of resuspension buffer and vortexed. The cells were then lysed by the addition of 200μl of lysis buffer and mixed by inversion. 150μl of neutralisation buffer was then added to precipitate the non-DNA material. The precipitate was pelleted by centrifugation at 13,000rpm for five minutes and the supernatant removed to a fresh Eppendorf

tube. The plasmid DNA was then recovered from this solution via phenol-chloroform extraction and ethanol precipitation as described previously.

Minipreps provided enough DNA to allow restriction mapping and thus the orientation of inserted DNA. Samples of miniprep DNA were also used for sequencing using T7 and SP6 primers to read the poly-linker region and inserted DNA in both directions. Larger quantities of DNA from selected colonies was prepared using Midiprep of Maxiprep kits also from Promega. The principals of both these kits were identical to the Miniprep but with larger quantities of materials.

2.8.11: Transfection of COS-7 cells

COS-7 cells are fibroblasts derived from African Monkey green kidney cells that have been transformed with an origin-defective mutant of SV40. As a result of SV40 transformation they are ideal recipients to support the growth of the pcDNA3 plasmid. Several different electroporation protocols were used in an attempt to transfect the cloned CD69 DNA into the COS-7 cells. Each was assessed for transient protein expression after twenty-four hours via surface staining on flow cytometry. The most successful protocol was as follows. Adherent COS cells were trypsinised and then washed in fresh DMEM as previously described. The cells were then resuspended at $2x10^6$/ml in DMEM and 1ml was transferred to a 4mm path length BioRad electroporation cuvette. Plasmid containing the DNA for transfection was added at 50μg/ml and the contents mixed well. A BioRad electroporator (BioRad Gene Pulser Tm) was used at a capacitance of 500μF and voltage of 350V to provide an electrical discharge to the cells with a time constant of between 5-6ms. Cells were allowed to recover in 1ml of DMEM at room temperature for five minutes before being plated out on petri dishes in 10ml of DMEM.

2.8.12: Immunomagnetic Selection of Transfected Cells

COS-7 cells that showed transient expression of CD69 were recovered via immunomagnetic bead selection with Dynal beads. Briefly, cells that had been identified as being positive for surface CD69 expression, via FACS analysis, were washed and resuspended in 500μl of mouse-anti-human CD69 (2μg/ml) and

incubated at 4°C for one hour on a gentle rotator. Cells were then washed twice to remove unbound antibody and resuspended in 500µl of fresh DMEM. 10µl of Dynal Dynabeads ($4x10^8$ beads/ml) coated with sheep anti-mouse IgG antibody were then added and incubated on the rotator for one hour at 4°C. CD69 positive cells where then positively selected due to the adherence of magnetic beads. The tube of bead labelled cells was placed in a magnetic field for one to two minutes and CD69 positive cells were dragged to the side of the tube. Any CD69 negative cells remained in free suspension and were removed via aspiration. The labelled cells were washed five times and the magnetic selection repeated each time. The selected cells were then washed from the tube onto a petri dish for continued culture.

2.8.13: FACS Sorting

After several rounds of culture and immunomagnetic selection a population of stable CD69 positive transfected cells developed. This population was purified using Fluorescence Activated Cell Sorting. For FACS sorting the cells were prepared as for staining except that the volume of antibody was increased with accordance to the number of cells involved and that sterile technique was used. Flow cytometry is prepared to assess the degree of staining in the population and the cells that are to be recovered are gated in respect to their FL-1 fluorescence. The gated population is then deflected into a collection tube due to droplet charge. 100,000 cells were collected per tube and then cultured for several days before being analysed again for surface CD69 expression.

2.9: STATISTICAL ANALYSIS

Where applicable all data is presented as the mean ± standard deviation (STDEV). In cases where the group of data covers a large range standard errors (SEM) are used instead of STDEV. Statistical significance was tested using Man Whitney or analysis of variance according to the distribution of the data.

CHAPTER 3

Effect of Na^+/K^+ATPase Modulation on Murine Models of Inflammation

3.1: INTRODUCTION

The Na^+/K^+-ATPase is an important membrane enzyme that plays a significant role in control of cellular volume and the cationic homeostasis of sodium and potassium. Inhibition of the Na^+/K^+-ATPase with ouabain results in dramatic effects on cationic homeostasis where the electrogenic flux of two potassium ions inwards and three sodium ions outwards is prevented. Consequently, a transient rise in intracellular sodium levels is observed. When a cell has its intracellular sodium concentration artificially raised, it may react using existing cation exchange mechanisms to maintain the sodium ionic homeostasis. Blaustein et al. (1976) (411) have described that downstream to rises in intracellular sodium, a concomitant rise in intracellular calcium can be detected, via an increase in the activity of the Na^+/Ca^{2+} exchanger.

Decreased Na^+/K^+-ATPase activity has been described in rheumatoid erythrocyte membranes have been described by Testa et al (1987) (412). Similarly, recent work from this laboratory has demonstrated decreased Na^+/K^+-ATPase activity in rheumatoid mononuclear cell membranes, via the use of a histochemical assay of PBMCs (243). If this defect in Na^+/K^+-ATPase activity is mimicked in healthy PBMCs via the use of the specific Na^+/K^+-ATPase inhibitor ouabain profound effects on cytokine production are observed. Newton (1990) (219) reported ouabain induction of PBMC IL-1β production and work carried out by Foey, in this laboratory, has complemented this by showing that ouabain stimulates IL-1β and TNFα production and suppresses IL-6 production from PBMCs, whilst inducing IL-6 production by synovial fibroblasts (232,413). This cytokine profile is similar to that described in RA and it is the hypothesis of this study that defective Na^+/K^+-ATPase activity in RA mononuclear cell membranes, perhaps due to oxidation of sulphydryl moieties, contributes to the pathogenesis of disease. Consequently, in an attempt to complement in vitro cytokine data, experiments were conducted in murine models of inflammation to assess the immuno-modulatory potential of ouabain in vivo.

In order to address the role of the Na^+/K^+ATPase in the development of murine arthritic disease it was first necessary to define a method for the measurement of ATPase activity. The ability of ouabain to inhibit murine Na^+/K^+ATPase activity had to be proven in order to validate the use of this compound in the animal models.

Several assays for the measurement of Na^+/K^+-ATPase activity have been previously described. The ATPase assay used previously in this laboratory was based upon that described by Chayen et al. (414). It relied upon the densitometric measurement of the formation of lead sulphide in tissue sections or slide mounted cell preparations. In a hope to develop an assay with improved sensitivity the ATPase assay described by J. M. Hamlyn (415) was employed. In this assay the regeneration of enzymatically hydrolysed ATP is coupled to the oxidation of NADH (see **method 2.3**). As NADH absorbs UV light at 340nm measuring the absorbance of an assay cocktail, containing NADH, at 340 nm can monitor the activity of total ATPase. The UV absorbance decreases in proportion to the oxidation of NADH therefore by calibrating the assay with exogenously added ADP, in the absence of cell lysate, it is possible to indicate the degree of ATPase activity (see **Figure 3.1A**). As the Na^+/K^+ATPase is ouabain sensitive the degree that this ATPase contributes to the oxidation of NADH can be measured by incubating the cells ± ouabain prior to initiating the assay. By subtracting the ouabain resistant activity from the total ATPase activity the Na^+/K^+ATPase activity can be defined.

3.1.1: Na^+/K^+ATPase Activity in Monocytes

The ATPase assay was initially developed using the human monocytic cell line THP-1. These cells provided a convenient source of plasma membrane, as they were large, grew rapidly and due to their non-adherence were easy to prepare. **Figure 3.1B** shows the effect of 200μM ouabain on total ATPase activity, indicated by the drop in UV absorbance of the assay cocktail, over a two hour period. From the two curves plotted in **figure 3.1B** it can be seen that the optimal time-point to measure the effect of ouabain under these conditions is approximately 60 minutes for THP-1 cells. After this time the ouabain resistant ATPases degrade the available ATP at a similar rate to the total ATPases in the control group. As a result of this, it appears that the optimal time to measure the Na^+/K^+ATPase activity is before the availability of ATP becomes the rate-limiting step of the reaction. Consequently this became an important consideration and preliminary studies on all different cell types included a detailed time-course of total ATPase activity. After defining the rate of the reaction for each cell preparation, a suitable time-point was chosen at which to determine Na^+/K^+ATPase activity. In THP-1 cells, suspended at 10^6/ml, a 60-minute assay

incubation was used for optimal Na^+/K^+ATPase activity determination. **Figure 3.2A** shows a ouabain concentration response curve for its effect on ATPase activity in THP-1 cells using these conditions. Optimal inhibition was seen at between 100μM and 200μM with 28% of total ATPase activity being ouabain sensitive. Similar procedures were carried out to define the ouabain sensitivity of the Na^+/K^+ATPase in different cell types and the degree to which the Na^+/K^+ATPase contributed to total ATPase activity. **Figure 3.2B** summarises these data and highlights the differences between murine and human monocytes. From this graph it can be seen that THP-1 cells have the highest level of Na^+/K^+ATPase activity with human PBMCs displaying a ouabain sensitive fraction of approximately 14% of total ATPase activity. When murine cells were compared to human cell types in this assay it was found that their Na^+/K^+ATPase was indeed less sensitive to ouabain, with ouabain concentrations of 400μM being required to cause maximal inhibition. In addition, it was seen that Na^+/K^+ATPase contributed a lesser percentage to total ATPase activity in both murine peritoneal macrophages and the murine monocytic cell line P388-D1. From **figure3.2B**, it can be seen that in P388-D1 cells 6% of total ATPase activity is attributable to Na^+/K^+ATPase activity and in peritoneal macrophages Na^+/K^+ATPase activity accounts for a similar 8% of total ATPase activity. Interestingly, peritoneal macrophages taken from animals that had received 34μg/kg ouabain daily for 14 days (Treated PMphage) showed no difference in total or ouabain sensitive ATPase activity, when compared to control mice.

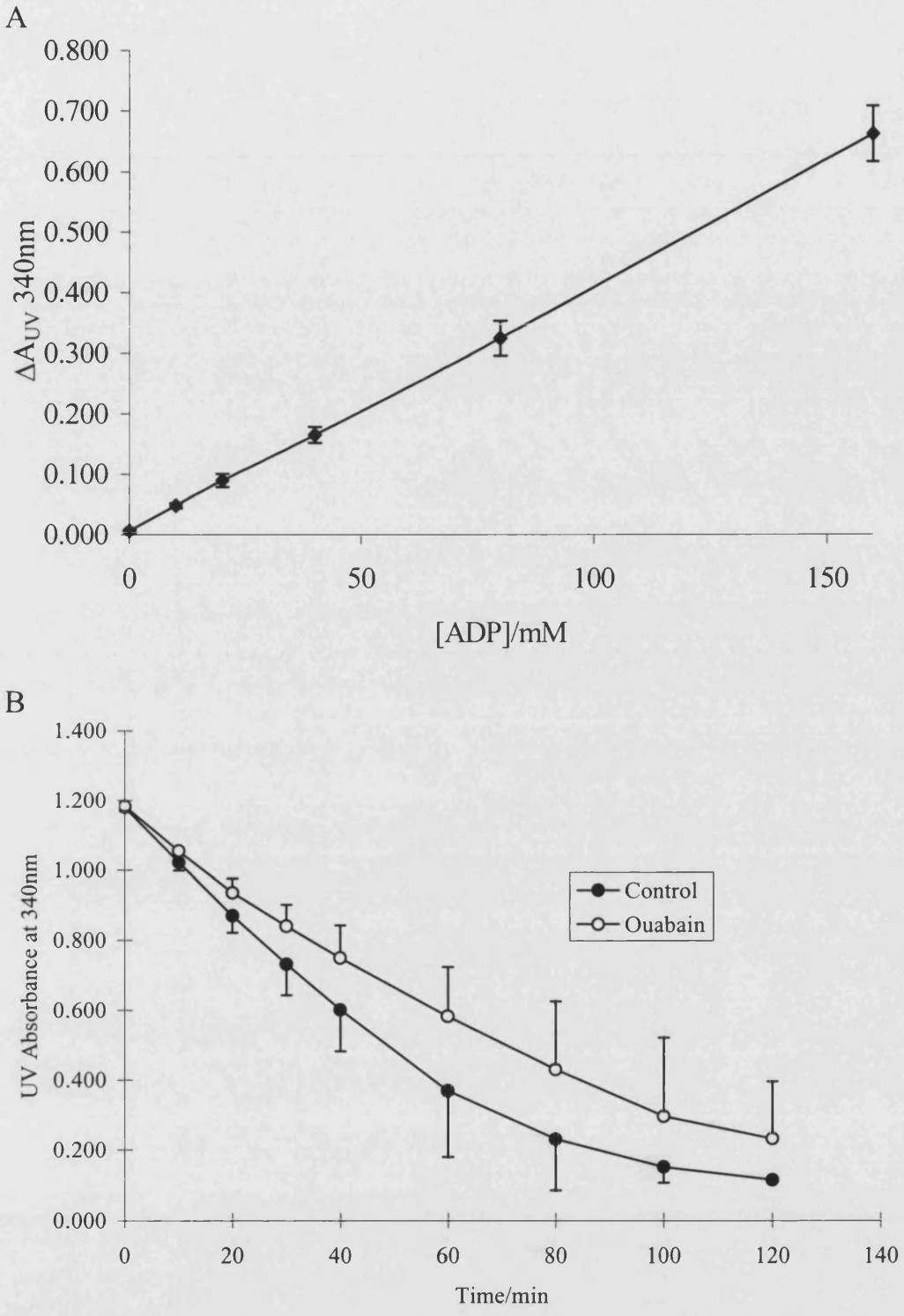


Figure 3.1: Determination of Na^+/K^+-ATPase Activity Using ATPase Assay.
Panel A: ADP calibration curve for the ATPase assay cocktail. Results represent the mean of three cocktail sample readings ±STDEV.
Panel B: Effect of 200μM Ouabain on ATPase activity in THP-1 cells (n=3±STDEV).

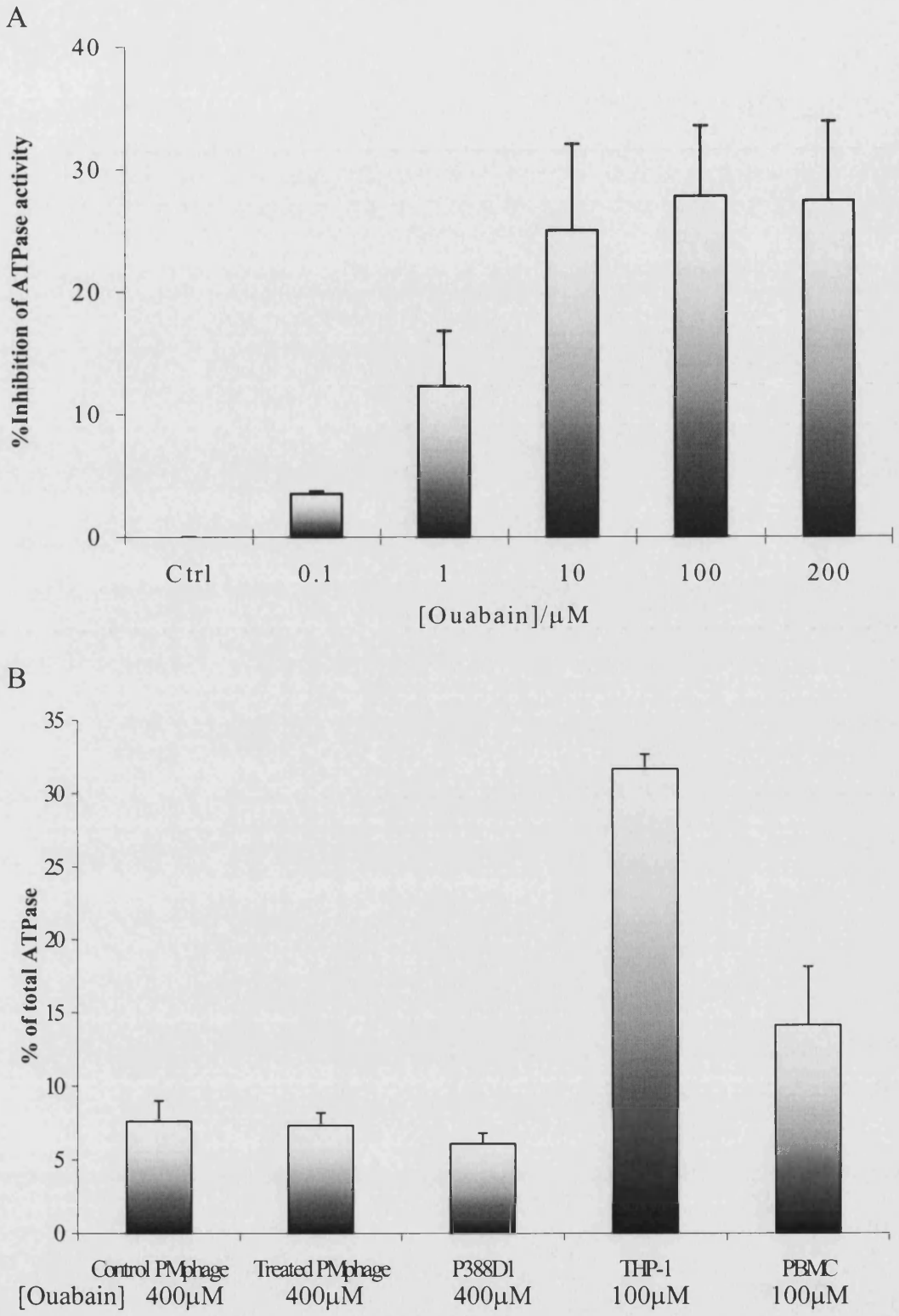


Figure 3.2: Inhibition of Na^+/K^+-ATPase in Different Cell Types.
Panel A: Concentration response curve for ouabain activity in THP-1 cells. Results indicate the percentage of total ATPase activity that is due to Na^+/K^+ATPase activity (n=3±STDEV).
Panel B: The percentage of total ATPase activity in various cells that is sensitive to inhibition by ouabain. Murine peritoneal macrophages were taken after 14 days of ouabain treatment (34μg/kg i.p.) or saline control (n=3±STDEV).

3.2: ANTIGEN INDUCED ARTHRITIS

3.2.1: Introduction

Due to sponsorship links with Knoll Pharmaceuticals the opportunity arose to investigate the effects of ouabain in animal models of arthritis. Although at this point detailed examinations of the effect of ouabain on human mononuclear cell cytokine production had not been completed, based on earlier work from this laboratory it was felt that animal studies could well be very profitable. Time limitations ruled out certain rabbit models and initial studies investigated the effects of ouabain on antigen induced arthritis in mice.

There was however, some doubt concerning murine ouabain resistance, a phenomenon which is not discussed greatly in previous literature as the main animal mentioned in conjunction with ouabain resistance is the rat. There was little time for detailed study of the murine Na^+/K^+ATPase and in the light of work on ouabain induced IL-1β release from human PBMCs (219,232) it was hoped that even a modest effect from ouabain may lead to an appreciable exacerbation of arthritis.

In hypertension studies it has been shown that ouabain, given acutely, does not produce a blood pressure change in the rat (416) but does so in sensitive species such as guinea pigs. Long term studies, however, with chronic administration of 14μg/kg/day ouabain in rats resulted in a significant increase in mean blood pressure after 6-8 weeks (417). This evidence gave hope that long-term effects of ouabain in mice would be evident in the arthritis model.

In human cells, the ATPase assay has shown the Na^+/K^+-ATPase to account for approximately 25-30% of the total ATPase activity. In mice however, using P388-D1 cells and peritoneal macrophage cells, the Na/K-ATPase was shown to account for only 5-10% of the total ATPase activity. The insensitivity of the Na/K-ATPase required ouabain concentrations of over 400μM for a measurable effect.

Although the ouabain sensitive portion of total ATPase activity was small in murine cells these experiments displayed potential for ouabain effects in the models of arthritis. However, the concentration of ouabain that had a measurable effect in vitro would not be tolerated by the animals so the consequence of long-term low dose treatment was critical to the in vivo results. As initial experiments were started as soon as possible a short murine toxicology study was carried out and 34μg/kg

ouabain dose determined to be safe for the animals. The insensitivity of the murine Na^+/K^+ATPase to ouabain, demonstrated in vitro with the ATPase assay, did however prompt a second toxicology study and the initiation of a further set of antigen induced arthritis models using 170μg/kg ouabain.

3.2.2: Disease Assessments

Uptake of ^{99m}-technetium can be used to quantify the severity of joint inflammation (418) and correlates well with inhibition of cartilage proteoglycan synthesis and chondrocyte death up to day 28. Also, serum levels of the acute phase protein serum amyloid P-component (SAP) correlate with the inflammatory changes in the joint during the acute phase of the arthritic response (days 0-7 after intra-articular challenge); however, during the chronic phase (day 7 onwards), serum SAP levels are unrelated to the extent of joint damage (419). Serum anti-BSA antibodies are readily detectable, but their measurement may be of limited value in that the steroid prednisolone, while markedly suppressing both synovitis and cartilage/bone erosions, did not reduce serum anti-BSA antibody levels (420).

As yet, histological observations provide the best means of disease assessment in this model. The slides are prepared as described in **method 2.4.1.1**, with samples being taken for sectioning on days 7, 14, 21 and 28.

3.2.2.1: Synovial Changes and Bone Erosion

Changes in the synovium are scored on a scale of 0-5 to take into account synovial lining cell hyperplasia, infiltration of the subsynovium by inflammatory cells, and pannus formation.

Bone erosions are scored on a scale of 0-5 to express the extent of cartilage and bone destruction, particularly focusing on the articulating cartilage of the femur and tibia. In some joints, more than 50% of these surfaces may eventually become eroded.

Sections from each test are scored by at least two independent observers with the result being the mean value of both scorers. The score given for each parameter is the mean value taken from both sections from each animal. Although a similar scoring system can be derived to assess the cellular content of the synovial fluid (mainly PMNs), it is found that the intra-group scores for this parameter show wide variations, which may, in part, reflect loss of synovial fluid during tissue processing.

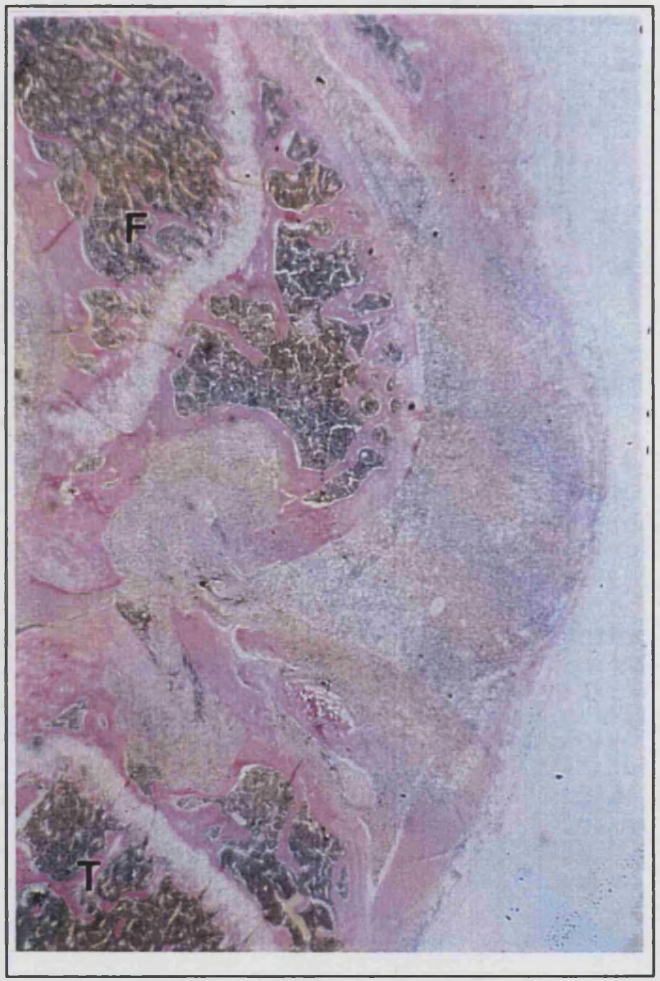
3.2.3: Synovial Joints: a guide to the joint histology displayed in the photographs

The articulating bony surfaces have at their ends a thin plate of dense cortical bone, known as the articular end-plate. Beneath this lies the cancellous bone often containing red (hematopoietic) marrow, and tightly adherent to the bony end plate is the hyaline articular cartilage (bearing and gliding surface).

Within the joint capsule and defining the inter-articular space is a specialised layer of connective tissue cells, the synoviocytes, which secrete the synovial fluid. Deep to this layer are varying amounts of highly vascular adipose, fibrous or areolar tissue supporting the synoviocytes and allowing the sac to be appropriately loose in certain ranges of motion, without allowing the synovial folds to become entrapped between the joint surfaces during movement. The synovial tissue has a rich blood supply to maintain the synoviocytes and also to provide the fluid they require. It also has numerous nerve endings that along with those in the capsule and spindles in the muscles, ligaments, and tendons, are responsible for the keen proprioceptive sense and deep pain perception that protect the joints.

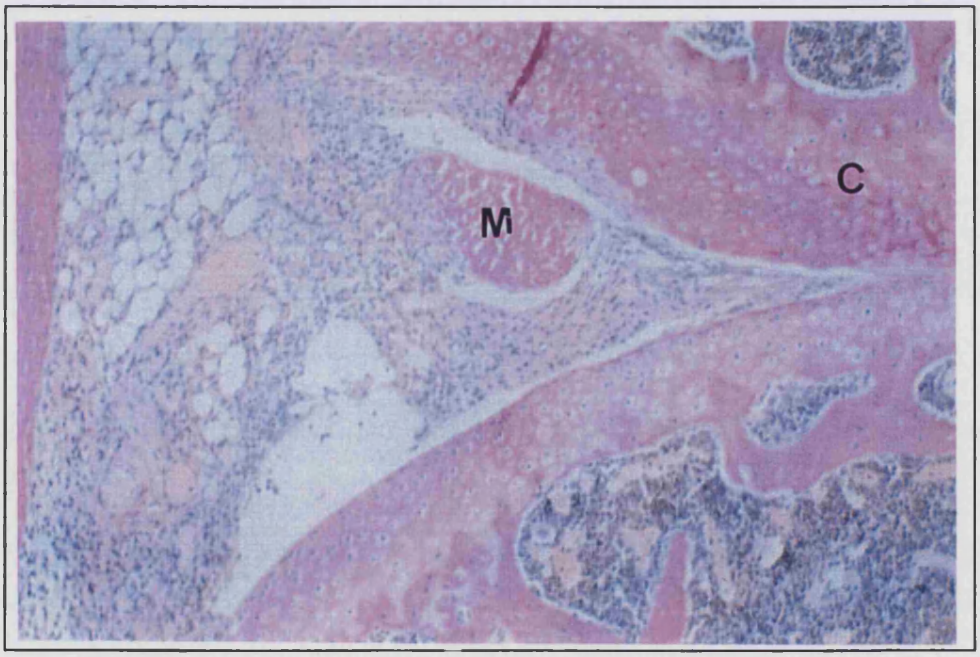
Certain of the bodies joints have within their cavities complete or, more often, incomplete fibrocartilaginous discoid partitions known as menisci. The menisci are highly developed and well defined in the knee and can be seen as triangular pink/red forms either side of articulating contact point. Synovium does not cover the avascular, aneural fibrocartilaginous menisci, which are firmly fixed to the joint margin by attachment to bone and to ligaments or capsule, preventing abnormal movement or intra-articular displacement during joint function. The function of menisci varies from site to site but generally involves stability.

Control Healthy Joint: Femur (F), tibia (T), meniscus (M) and growth plate (G).



Inflamed Joint: Showing bone erosion and synovitis. Femur (F) and tibia (T).

High power section showing synovitis. Synovial cavity (S) and articulating cartilage (C).



High power section showing synovitis and early bone erosion. Meniscus (M) and articulating cartilage (C).

3.3: RESULTS

In total, four antigen induced arthritis (AIA) experiments were carried out. The two groups of two experiments compared the effects of 34μg/kg and 170μg/kg ouabain on antigen induced arthritis in animals immunised with or without *B. Pertussis*. In each experiment seven animals were taken at each time point (days 7, 14, 21 and 28) and the slides prepared of the histological samples were scored on a 1-5 scale for synovitis and bone erosion. In all cases a severe synovitis was observed, as early as day 7, which gradually became less severe over the 28 day study. Conversely, very little bone erosion was observed on day 7 but over the 28 day period cartilage degradation and erosion of the underlying bone became more apparent (see **figure 3.3A**).

Figure 3.3 also shows a comparison between the groups immunised with (**A**) and without (**B**) *B. Pertussis*. Immunisation with *B. Pertussis* had no significant effect on the development of bone erosion but resulted initially in slightly greater levels of synovitis. The two different models did appear to give subtly different conditions in which to test the effects of ouabain but both were quite severe forms of arthritis, with high levels of cartilage destruction. **Table 3.1** summarises the data of all four experiments, including that shown in **figure 3.3**, and shows that at no point did ouabain display any significant effect on the severity of disease. However, there was a trend for ouabain to cause a slightly higher degree of bone erosion in the experiments without immunisation with *B. Pertussis.* Also, on day seven after disease induction there was a slightly higher level of synovitis in these experiments. This effect was not statistically significant but did occur with both concentrations of ouabain studied and offered some evidence of a possible effect of ouabain in these models. It remained to be seen whether the effect of ouabain may just have been overwhelmed by the severity of the arthritis in this model.

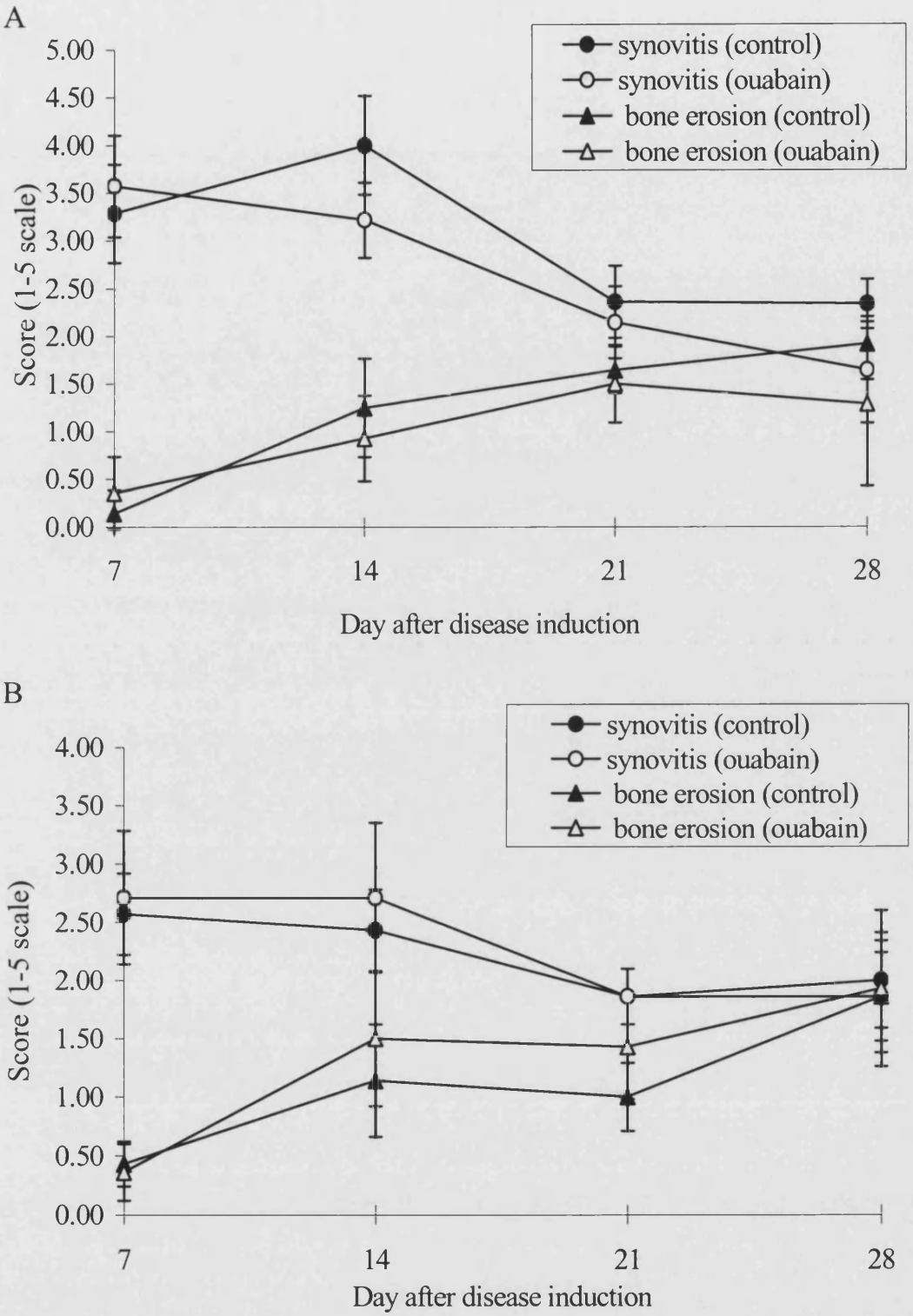


Figure 3.3: Effect of Ouabain on Murine Antigen Induced Arthritis (AIA).

Panel A: AIA in animals immunised with B. pertussis. Joints were taken for histological analysis on various days after disease induction. Ouabain treatment (170μg/kg i.p.) commenced two days prior to disease induction. Scores represent mean of two independent observers (n=7± STDEV).

Panel B: AIA in animals immunised without B. pertussis. The ouabain treatment and disease assessment were carried out as in figure A except animals were not immunised with B pertussis prior to disease induction (n=7± STDEV).

34μg/kg Ouabain: Immunised with pertussis.

	Synovitis				Bone Erosion			
Day	control	STDEV	ouabain	STDEV	control	STDEV	ouabain	STDEV
7	2.43	0.67	2.5	0.41	0.21	0.27	0.21	0.27
14	1.92	0.38	2	0.65	0.83	0.26	0.79	0.27
21	1.79	0.27	2	0.29	1.5	0.5	1.71	0.49
28	1.93	0.45	1.71	0.27	1.79	0.7	1.5	0.29

34μg/kg Ouabain: Immunised without pertussis.

	Synovitis				Bone Erosion			
Day	control	STDEV	ouabain	STDEV	control	STDEV	ouabain	STDEV
7	2.14	0.94	2.86	0.38	0.29	0.39	0.64	0.24
14	2	0.38	1.57	0.61	1	0.38	0.71	0.39
21	1.29	0.39	1.36	0.48	0.71	0.39	0.86	0.63
28	1.36	0.38	1.14	0.68	1.07	0.61	0.36	0.38

170μg/kg Ouabain: Immunised with pertussis.

	Synovitis				Bone Erosion			
Day	control	STDEV	ouabain	STDEV	control	STDEV	ouabain	STDEV
7	3.29	0.52	3.57	0.53	0.14	0.24	0.36	0.38
14	4.00	0.52	3.21	0.39	1.25	0.52	0.93	0.45
21	2.36	0.38	2.14	0.38	1.64	0.24	1.50	0.41
28	2.33	0.26	1.64	0.56	1.92	0.38	1.29	0.86

170μg/kg Ouabain: Immunised without pertussis.

	Synovitis				Bone Erosion			
Day	control	STDEV	ouabain	STDEV	control	STDEV	ouabain	STDEV
7	2.57	0.35	2.71	0.57	0.43	0.19	0.36	0.24
14	2.43	0.35	2.71	0.64	1.14	0.48	1.50	0.58
21	1.86	0.24	1.86	0.24	1.00	0.29	1.43	0.45
28	2.00	0.41	1.86	0.38	1.86	0.48	1.93	0.67

Table 3.1: Summary of all Antigen induced Arthritis data. Each of the four tables represents a separate experiment of 28 animals with the treatment conditions detailed in the legend. Data represents the mean assessment scores for seven animals at each time point.

3.4: ZYMOSAN INDUCED ARTHRITIS

3.4.1: Introduction

Zymosan induced arthritis was first described by Keystone et al. 1977 (64). Like antigen induced arthritis it is reported to be an IL-1 dependent inflammatory disease localised to the joint by intra-articular administration of the inducing agent, in this case Zymosan A (*Saccharomyces cerevisiae*). In contrast to antigen induced arthritis, however, zymosan induced arthritis does not rely upon an adaptive immune response but rather the innate inflammatory reaction. Consequently, a less chronic inflammatory condition occurs in the joint, giving a model that may be more sensitive to pro-inflammatory treatments and thus allow the detection of the effects of ouabain.

In these experiments a histological study was carried out, with slides being prepared as in the antigen induced arthritis, to investigate the gross effects of the inflammatory response. The less severe nature of this model also allowed a more sensitive assay of biochemical changes within the joint to be used, namely a proteoglycan (PG) synthesis assay in which the incorporation of ^{35}S [SO_4] into the sulphated moieties of the proteoglycan monomers is monitored. Studies of the metabolism of articular cartilage demonstrate a surprising rate of synthesis and degradation of the component matrix materials. The rapid turnover of at least a small portion of the proteoglycan suggests the presence of an internal remodelling system, and evidence has accumulated that this system is based on the release of lysosomal enzymes from the chondrocytes, which have as their principal substrate the proteoglycan. Activity of this remodelling system is modulated by various cytokines and growth factors.

The physicochemical properties of PG/hyaluronic acid aggregates within the extracellular matrix confer upon articular cartilage its elastic properties. Under normal conditions, endogenous cartilage cells (chondrocytes) regulate the synthesis and matrix levels of PG. Recent studies have shown that the two main factors regulating PG production are insulin-like growth factor-1 (IGF-1) (421) and transforming growth factor β (TGFβ) (422). These factors mediate their effects via specific receptors on the chondrocyte cell membrane. The observation that chondrocytes are able to synthesise both IGF-1 (423) and TGFβ (521) suggest potentially important autocrine functions for these factors.

It has long been recognised that depletion of the extracellular matrix components of articular cartilage, specifically proteoglycan (PG) and collagen, are pivotal events relating to restriction or loss of joint function in patients with rheumatoid or osteoarthritis, and in animal models of these diseases. If depressed Na^+/K^+ATPase activity in the mononuclear cells of the mice in this study contributes to the severity of arthritis then PG synthesis measurement would offer a clearly quantifiable method of detection that is relevant to human disease.

In inflammatory conditions, particularly RA, chondrocyte function may be modulated by cytokines derived from cells of the inflammatory lesion. IL-1 in particular appears to be of paramount importance as a cartilage catabolic factor. In vitro, IL-1 has been shown to both inhibit PG synthesis and stimulate PG degradation in cartilage of either animal (424) or human (425) origin. In vivo, IL-1 injected into either rabbit or rat knee joints, induced cartilage proteoglycan depletion in the challenged joints concurrent with an increase in synovial fluid PG levels (426). Furthermore, in mice intra-articular injection of murine recombinant IL-1α and IL-1β suppressed chondrocyte PG synthesis whereas TNF only marginally affected PG synthesis at the highest dose used (427). Treatment of zymosan induced arthritis with anti-IL-1(α+β), but not with anti-TNF, resulted in normal PG synthesis, confirming the key role played by IL-1 in the inhibition of PG synthesis (428).

Evidence from studies in vitro has suggested that both IGF-1 (429) and TGFβ (523) are able to counteract the effects of IL-1 both on PG synthesis and degradation.

In this study zymosan induced arthritis provides a model whose inflammatory effects have been shown to be highly dependent upon IL-1. By measuring PG synthesis in the patella as an indicator of the degree of inflammation, the consequences of changes in IL-1 levels within the joint should be determinable. The effects of ouabain upon the degree of induced inflammation, which were perhaps lost in the severity of antigen induced arthritis, may become detectable. The sensitivity of the PG synthesis assay to IL-1 modulation should show any ouabain-induced abnormalities in murine mononuclear cell production of IL-1.

This study also investigated the effect of ouabain upon IGF-1 stimulated PG synthesis. In-house studies at Knoll had shown that both basal and IGF-1 stimulated PG synthesis were significantly impaired in arthritic patellar cartilage compared with their non-arthritic controls. This diminished response to IGF-1 was also induced in

normal patellae by exposure to IL-1. In both instances, IGF-1 mediated PG synthesis was inhibited before any change in basal PG synthesis was seen. This evidence indicates that IGF-1 stimulated PG synthesis is more sensitive to IL-1 levels than is basal PG synthesis therefore providing an even more sensitive system for the detection of ouabain induced IL-1 production.

3.5: RESULTS

3.5.1: Effect of Zymosan Induced Arthritis on Joint Pathology and PG Synthesis

The zymosan induced arthritis model was characterised using histological sectioning to compare it to the antigen induced arthritis model. As can be seen in **figure 3.4A** the degree of synovitis and bone erosion was much lower in zymosan induced arthritis than it was in antigen induced arthritis (**Figure 3.3**). Although on day 4 the synovitis appears to be worse in the ouabain group, the error involved renders this insignificant. Histologically ouabain (34μg/kg) had no effect on either synovitis or bone erosion in this model, but this was not surprising due to the short duration of the model inducing little joint pathology.

Initial experiments showed quite low incorporation of ^{35}S [SO_4] into the PG of both arthritic and control patellae so in an effort to increase the availability of ^{35}S [SO_4] in the incubating media the unlabelled SO_4 content was reduced by 50%. Labelled sulphate incorporation improved and showed a significant contrast between arthritic and control PG synthesis. The model consistently demonstrated that arthritic patellae had approximately a three-fold decrease in PG synthesis when compared to contralateral control patellae (**figure 3.4B**). Another interesting trend was the increased PG synthesis observed in arthritic patellae during the later stages of the model. Although the magnitude of this increase in PG synthesis was not always statistically significant, this trend appeared in every experiment and offered another window of opportunity for observing effects due to ouabain. It could be predicted that a worse inflammatory disease state would lead to a greater degree of rebound tissue repair.

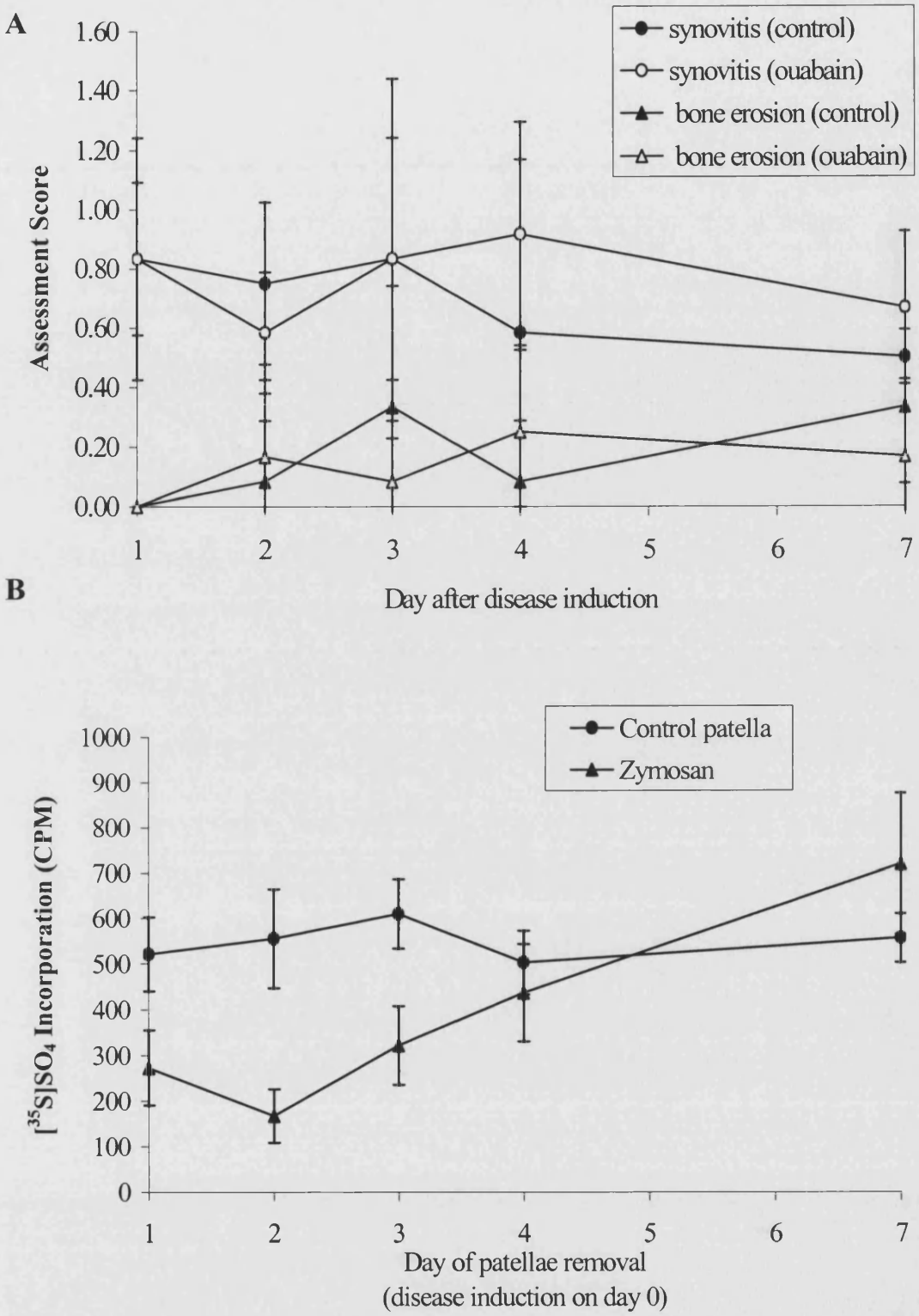


Figure 3.4: Zymosan Induced Arthritis (ZIA): Histological Analysis Compared to Biochemical Analysis.

Panel A: Histological analysis of ZIA in animals treated ± Ouabain (34µg/kg). Joints were taken for analysis on various days after disease induction. Ouabain treatment commenced two days prior to disease induction. Scores represent mean of two independent observers (n=6± STDEV).

Panel B: Effect of ZIA on PG synthesis in murine patellae (n=6± STDEV).

3.5.2: Effect of Ouabain on PG Synthesis

Mice were pre-treated with ouabain at 34μg/kg/day and 170μg/kg/day for two days prior to the initiation of arthritis, as described in **method 2.4.2**. Initial experiments quantified PG synthesis on days 1, 2, 3, 4 and 7 after disease induction in order to observe the severity and duration of the arthritis. At each time-point patellae were taken from 6 mice, with arthritic joints being compared to contra-lateral controls. The incorporation of ^{35}S $[SO_4]$ during three hours of incubation being measured. As can be seen in **figure 3.5A**, ouabain (34μg/kg) significantly inhibited the recovery of PG synthesis with day three patellae still having severely depressed ^{35}S $[SO_4]$ incorporation compared to the controls ($p=0.0039$). Also worthy of note is the slightly elevated, if not statistically significant, over-shoot in ^{35}S $[SO_4]$ incorporation on day seven.

Unfortunately, this was the only experiment that showed a significant effect of ouabain in this model. Several attempts were made to repeat this finding, two of them showed a trend for ouabain treated animals to have delayed PG synthesis recovery but the other experiments showed no effect for ouabain at all. **Figure 3.5B** shows the collated results for all these later experiments representing data from 18-30 animals per time point.

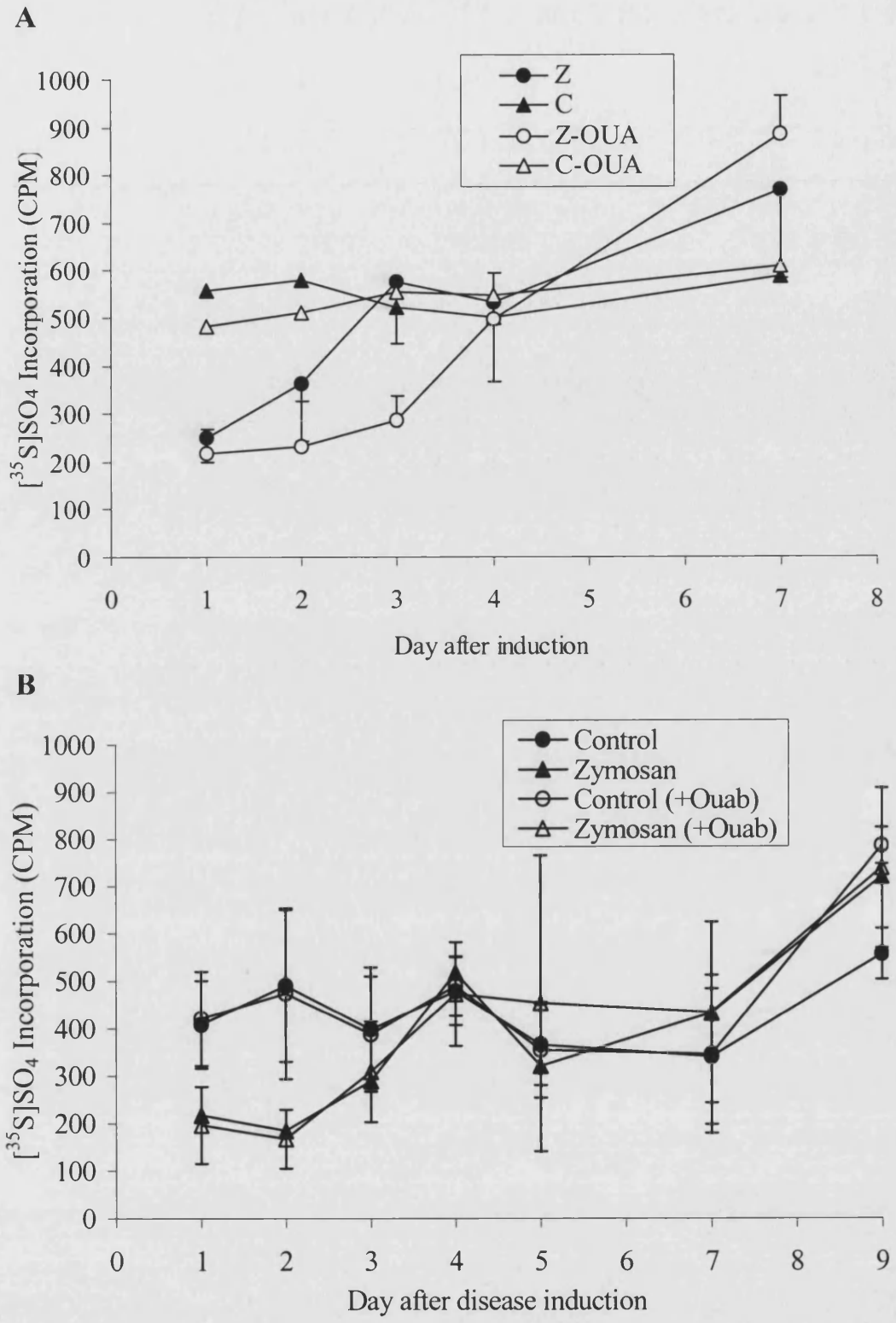


Figure 3.5: Ouabain Modulation of PG Synthesis in Arthritic and Normal Murine Patellae.

Panel A: Effect of ouabain (34μg/kg) on recovery of PG synthesis in murine patellae over remission period of ZIA (** = $p<0.005$) (n=6 ± STDEV).

Panel B: Effect of ouabain (170μg/kg) on recovery of PG synthesis in murine patellae over remission period of ZIA (n=18-30 ± STDEV).

3.5.3: Effect of IGF-1 Stimulation on PG Synthesis

IGF-1 stimulated PG synthesis has been previously shown, in studies at Knoll, to be more sensitive to IL-1 inhibition than basal synthesis. Consequently, it may be expected that any small change in IL-1 levels in the joint due to ouabain treatment may be more easily detected. Before ouabain could be used in a model of IGF-1 induced PG synthesis various aspects of the model had to be defined. Firstly, an IGF-1 concentration response curve was constructed to compare the response of arthritic and control patellae. Patellae were incubated for 24hrs with IGF-1 and the level of $^{35}S[SO_4]$ incorporation was measured over the last three hours. Control patellae showed an almost two-fold increase in PG synthesis compared to arthritic patellae (Day 1 after disease induction), with a maximal stimulus occurring at 150ng/ml IGF-1 (see **figure 3.6A**). However, at no concentration was IGF-1 able to reverse the inhibition of PG synthesis observed in the arthritic patellae.

Also, to confirm previous reports from Knoll pharmaceuticals, a short study was performed with normal patellae in which an IGF-1 concentration response curve was challenged with a single dose of IL-1β. As can be seen from **figure 3.6B** IGF-1 induced $^{35}S[SO_4]$ incorporation was similar to that seen in the normal patellae in the previous study (**figure 3.6A**). Incubation of the patellae with 0.75ng/ml IL-1β resulted in a reduction of PG synthesis to levels which were approximately three-fold lower than those seen in the control patellae. Basal PG synthesis was also inhibited by IL-1β but not as significantly as IGF-1 induced PG synthesis. Interestingly, this concentration of IL-1β inhibited IGF-1 induced PG synthesis in a similar but more potent manner than zymosan induced arthritis. Similarly to the arthritic patellae in the previous study, IL-1β treated patellae displayed a reduction in PG synthesis which was not surmountable with IGF-1. Even at the highest dose used, IGF-1 could only bring PG synthesis back to levels similar to those seen in untreated healthy patellae.

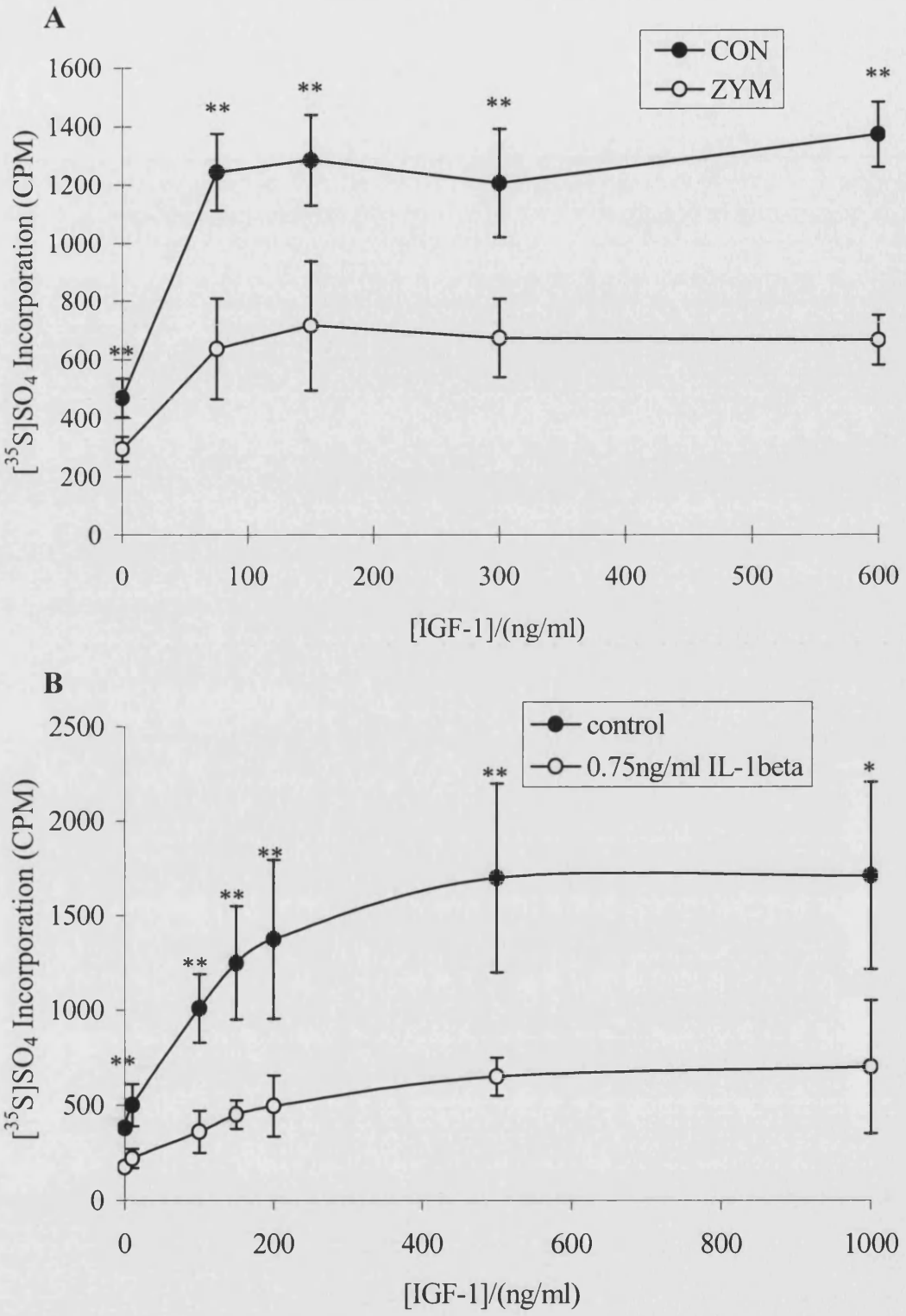


Figure 3.6: Effect of IGF-1 Stimulation on PG Synthesis in Murine Patellae.
Panel A: PG synthesis in arthritic and control patellae induced by IGF-1. Arthritic patellae were taken form animals on day 1 after disease induction with Zymosan and responses were compared to contra-lateral controls. IGF-1 was incubated with the patellae for 24 hours and $[^{35}S]SO_4$ was added for the last three hours of culture (n=6 ± STDEV, **=P<0.005)
Panel B: Effect of IL-1β on IGF-1 induced PG synthesis in normal patellae (n=6 ± STDEV, *=P<0.05, **=P<0.005).

3.5.4: Effect of Ouabain on IGF-1 Induced PG Synthesis

From the previous studies it was decided to use an IGF-1 concentration of 300ng/ml to stimulate PG synthesis in patellae taken from the zymosan induced arthritis model on days one, three and seven. Using these time-points it was hoped that the effect of ouabain on the response to IGF-1 could be studied whilst the inflammatory condition was at its most severe, during its recovery phase and then during the "remodelling" phase when PG synthesis was elevated.

Ouabain treatments and sample preparation were repeated as before. The patellae were incubated with IGF-1 for 24hrs, with $^{35}S[SO_4]$ incorporation being measured over the last three hours.

Firstly, the effect of IGF-1 stimulation was studied over the time-course of this model without considering the effect of ouabain. **Figure 3.7A** displays data comparing PG synthesis in control normal and arthritic patellae to IGF-1 stimulated normal and arthritic patellae. Day one clearly shows that the difference between the IGF-1 stimulated normal and diseased responses is far greater than that between control normal and diseased patellae. These data highlight the fact that IGF-1 stimulated PG synthesis is very sensitive to inhibition and complements the data, presented in the previous section, showing IL-1β inhibition of IGF-1 stimulated PG synthesis. Day three shows that in the control group arthritic patellae PG synthesis has recovered to equivalent levels seen in the normal patellae but that the IGF-1 response is still slightly reduced in the arthritic group. On day seven the same trends are seen in both the controls and the IGF-1 stimulated groups, with the arthritic patellae displaying higher levels of PG synthesis than the controls.

This model offered several aspects that could be affected by ouabain. However at no point was there any significant difference in the responses of control and ouabain treated mice. As can be seen from **figure 3.7B** ouabain had no effect on control or arthritic responses to IGF-1 on any of the three days.

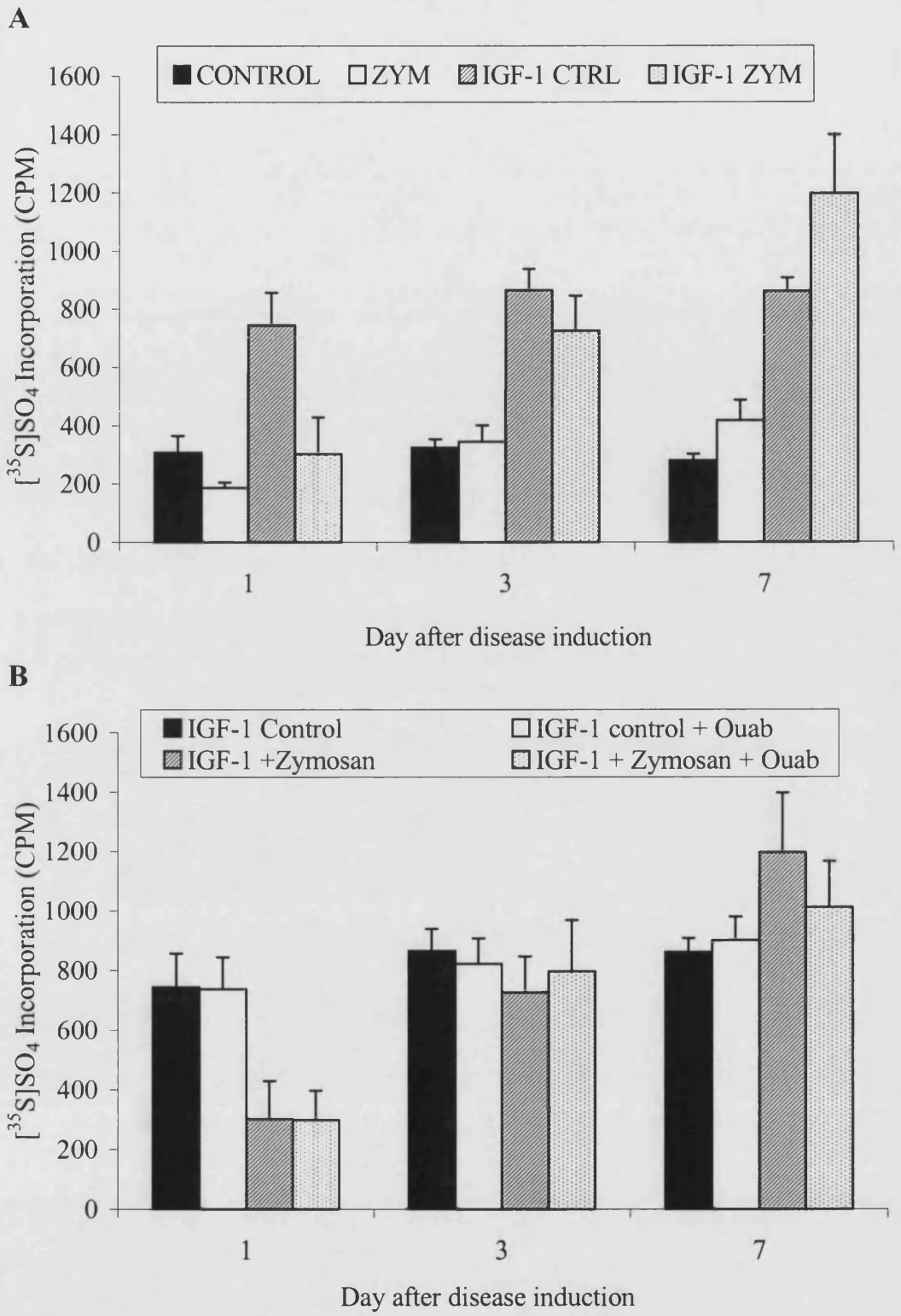


Figure 3.7: Responses of Arthritic Patellae to IGF-1 Induce PG Synthesis During Disease Remission Compared to Contralateral Controls.

Panel A: Variation in the responsiveness of patellae to IGF-1 induced PG synthesis during the progression of zymosan induced arthritis (n=6 ± STDEV).

Panel B: Effect of ouabain (34µg/kg) on IGF-1 induced PG synthesis in normal and arthritic patellae during the progression of zymosan induced arthritis (n=6 ± STDEV)

3.5.5: Zymosan Induced Arthritis in ICE$^{-/-}$ Mice

The results presented so far using the zymosan induced arthritis model have all been based upon the belief that the inflammatory condition induced in these animals is highly IL-1β dependent. Treatment of zymosan induced arthritis with anti-IL-1(α+β), but not with anti-TNF neutralising antibodies, has been shown to result in normal PG synthesis, confirming the key role played by IL-1 in the inhibition of PG synthesis (428). Despite this evidence, because of the availability of ICE$^{-/-}$ genetically modified mice a complementary study was set-up to assess the role of ICE in ZIA. There were, however, only six mice available (with control wild types) so the results are just for day one of the arthritis. Therefore, only the severity of disease induced is shown and not the recovery phase. As can be seen from **figure 3.8**, PG synthesis in the arthritic patellae of the ICE knockout mice was depressed to the same extent as that in the arthritic patellae of wild-type mice. These data indicate that even in the absence of mature IL-1β, zymosan raises a reaction within the murine joint that severely depresses the ability of chondrocytes to synthesise proteoglycans. Of course, based on such low numbers these data is only preliminary but even so, the inflammatory response in the ICE$^{-/-}$ mice could not be mistaken. These data question the importance of IL-1β in the pathogenesis of ZIA at 24 hr after disease induction and suggest the possibility that other cytokines, such as TNFα, may become dominant in the inflammatory response of ICE$^{-/-}$ mice.

Previous studies have shown that in mice intra-articular injection of murine recombinant IL-1α and IL-1β suppresses chondrocyte PG synthesis whereas TNFα only marginally affected PG synthesis at the highest dose used (427). Also, anti TNFα neutralising antibodies have little effect on the pathogenesis of ZIA (57). This suggests that in both the healthy joint and the ZIA-arthritic joint TNFα may not play a critical role in the regulation of chondrocyte PG turnover when IL-1β is also present. Whether TNFα is more important in the pathogenesis of ZIA in ICE-/- mice remains to be seen.

Out of curiosity, three of the ICE$^{-/-}$ animals were treated with 170μg/kg ouabain, as described before, but again there was no effect seen on the PG synthesis in any of the animals.

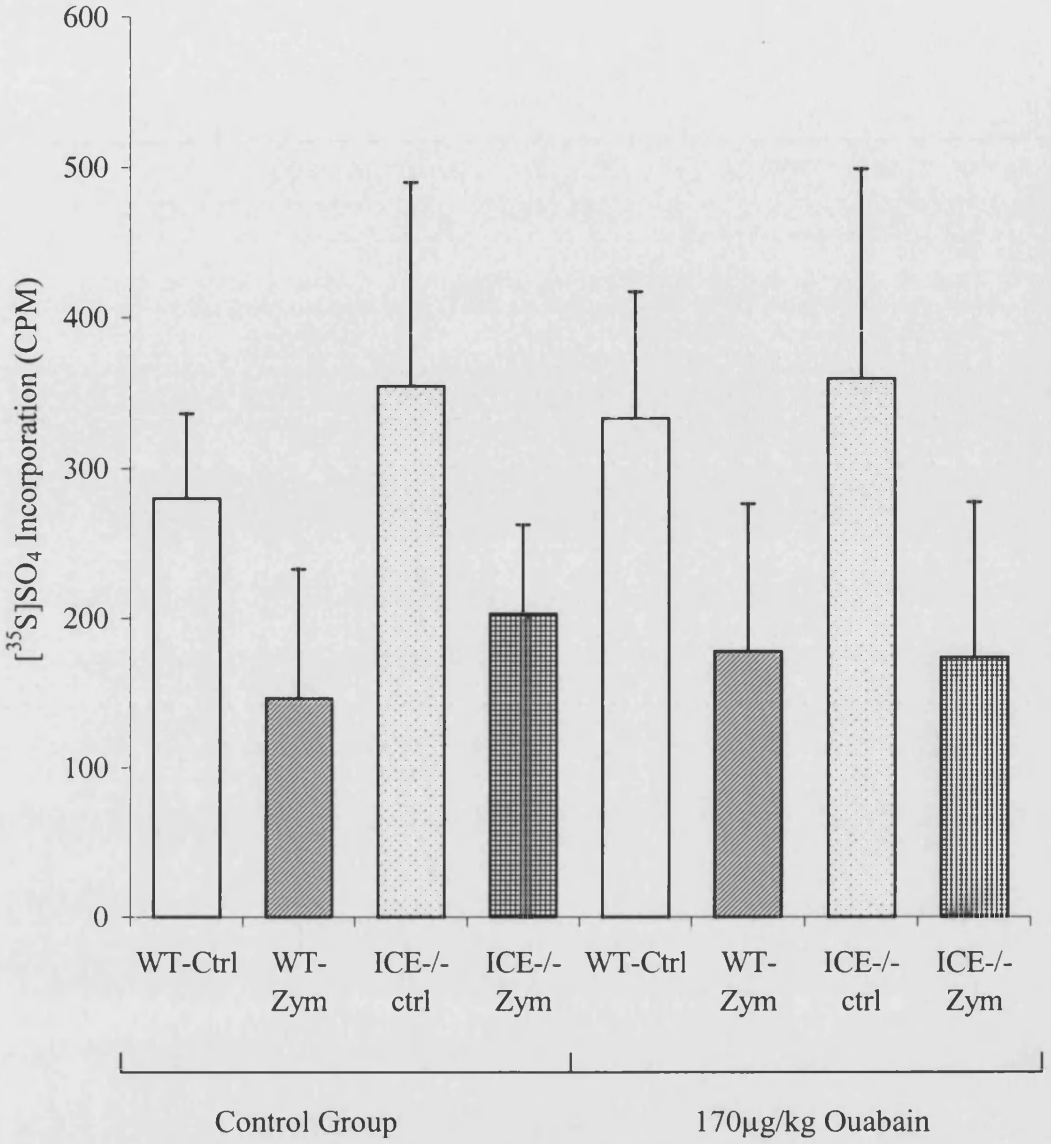


Figure 3.8: Effect of Zymosan Induced Arthritis on PG Synthesis in ICE$^{-/-}$ Mice.
PG synthesis was measured in ICE$^{-/-}$ knockout mice one day after zymosan induced arthritis. Responses were compared in Wild Type controls and the effect of ouabain on disease severity was also assessed (n=3 ± STDEV).

3.6: SUMMARY

This chapter has addressed some of the pathological features of murine models of arthritis and attempted to link evidence of abnormal Na^+/K^+ATPase activity with aberrant mononuclear cell activity in the inflamed joint. In summary, the results presented here have shown:

1. The development of an effective ATPase assay which allows the quantification of the ouabain sensitive Na^+/K^+ATPase component.
2. Any effects attributed to ouabain in the inflammatory response can not be illustrated using the murine model of antigen induced arthritis.
3. Models of arthritis which are more mild, such as murine zymosan induced arthritis, may be more suitable for the detection of small changes in the severity of inflammation but still show that ouabain doesn't show a consistent significant modulatory effect.
4. Murine ouabain resistance is sufficient to overcome any modulatory effects ouabain may have on inflammation.

Using the human monocytic cell line THP-1 an assay has been developed, based on that used by Hamlyn, to measure ATPase activity in cell lysates. This assay has shown that healthy cultured THP-1 cells have Na^+/K^+ATPase activity that accounts for 25-30% of total ATPase activity. The Na^+/K^+ATPase activity in these cells was totally inhibited with 200μM ouabain.

Evidence of ouabain resistant forms of the Na^+/K^+ATPase enzyme have been reported in rodents and ouabain resistance can be conferred to primate cells via transfection with a 6.4 kB fragment of murine genomic DNA (known as murine ouabain resistance gene) (430). In this study, however, evidence of large changes in IL-1β production in human PBMCs, induced by very low (nM) concentrations of ouabain, and reports on ouabain sensitivity in rats after long-term treatment fuelled interest in the possible effects of ouabain on in vivo models of inflammation.

The murine model of antigen induced arthritis has been used at Knoll Pharmaceuticals to study putative anti-rheumatic drugs. During the period of work carried out at Knoll for this report time constraints and animal availability proposed this model as being the most convenient in which to try and demonstrate pro-arthritic

properties of ouabain. Any doubts of murine ouabain resistance were disregarded in the hope that even a very small inhibition of the Na^+/K^+ATPase by ouabain would result in a measurable defect in mononuclear cell activity and detectable changes in the model of arthritis.

The ATPase assay demonstrated that murine cells were resistant to ouabain with peritoneal macrophages and the monocytic cell line P388-D1 indicating that only 5-10% of the total ATPase activity was due to Na^+/K^+ATPase activity. The concentrations of ouabain that were used to inhibit the Na^+/K^+ATPase were greater than 400μM with ranges up to 1mM being required for maximal inhibition. These results indicated that murine Na^+/K^+ATPase was about ten-fold less sensitive to ouabain than human Na^+/K^+ATPase. There was however potential for this degree of inhibition to be effective in the arthritis model but there was no way that concentrations of this kind could be attained in vivo. Recent in vivo studies in rats have shown that long-term treatment with ouabain (four weeks with 14μg/kg/day followed by four weeks at 28μg/kg/day) induced hypertension (417). The antigen induced arthritis model is a long term study (28 days) and levels of ouabain in excess of 14μg/kg could be given to the mice. By increasing the dose of ouabain to 170μg/kg and by varying the disease induction by omitting pertussis toxin in some of the animals it was hoped to maximise the possibility of demonstrating pro-arthritic effects of ouabain.

Unfortunately ouabain had no effect on antigen induced arthritis in mice. All four studies carried out showed consistent inflammatory responses with active synovitis and bone erosion, but ouabain had no significant effect on either of these parameters.

It is possible that even without immunisation with pertussis toxin this model develops such a severe arthritis that any modulatory effects due to ouabain would be lost in a flood of pro-inflammatory cytokines. To try and counteract this problem the milder, non-immune model of zymosan induced arthritis was used.

In zymosan induced arthritis the hosts defence involves the innate immune response and not the adaptive immune response. Consequently a temporary, resolving condition is induced with measurable synovitis but little obvious bone erosion over the short period of each study. The innate response still relies heavily upon the

production of IL-1β from infiltrating mononuclear cells so any aberrant mononuclear cell activity due to ouabain treatment should be demonstrable.

Histologically ouabain had no effect on zymosan induced arthritis in mice. The measurement of synovitis and bone erosion over the seven-day period revealed a very mild inflammatory condition with quite large intra-group variations in disease assessments. In order to provide a more sensitive measure of whether ouabain treatment can modulate the inflammatory reaction proteoglycan synthesis in patellar cartilage was monitored. Even if ouabain did not cause a more severe inflammation this assay would provide a sensitive way of watching the recovery of the joint after disease induction and provide data on the ability of ouabain to prolong joint pathology. Again, IL-1β is of major importance in this hypothesis as very low doses incubated with patellae have been shown to almost totally inhibit PG synthesis (428). Depleted PG synthesis in arthritic patellae has also been shown to be reversible with anti-IL-1β antibodies and IL-1ra treatment (428).

In this study ouabain showed its first positive signs of being pro-inflammatory. At 34μg/kg ouabain treated animals showed significantly depressed PG synthesis on day three of the disease when compared to controls and a possibly higher reflex increase in PG synthesis on day seven. Unfortunately, this result was not reproducible and subsequent experiments showed trends towards pro-inflammatory activity for ouabain if anything at all. From these studies it was noticeable that the positive result and the trends towards pro-inflammatory activity had been shown in models that displayed conditions which showed improvement on day two for all groups. The negative results were in models that displayed a condition that worsened on day two. This evidence again indicated that the effects of ouabain may be being lost in the severity of the model and further attempts to improve assay sensitivity were made.

IGF-1 stimulation of PG synthesis was used to enlarge the window in which to observe the effects of ouabain. IGF-1 stimulated PG synthesis has been shown to be more sensitive to IL-1β levels than basal synthesis therefore small changes in IL-1 levels are more easily detectable. Again ouabain was seen to have no effect although only one of the two experiments gave definable results.

Out of interest a small study at this point was carried out in ICE knockout mice. In theory their inability to process IL-1β should mean that the degree of inflammation

induced by any of the above models is significantly less than that in control animals. This theory is based on the demonstrated IL-1β dependence of the models. Surprisingly, though, there was no difference between ICE knockout mice and wild type mice in the effects of zymosan induced arthritis measured by PG synthesis. In this study only three animals were used per group and measurements were only taken on day one of the model so no conclusions can be drawn on the progression of the disease model in the knockout mice. The initial severity of the PG synthesis inhibition however questions the importance of IL-1β in this model suggesting that there is a degree of redundancy within the pro-inflammatory cytokine family, with TNFα and IL-1α perhaps playing key roles. The study of IL-1β knockout mice in the zymosan induced arthritis model would clarify the role of IL-1β.

Further investigation of these models would be interesting but in the mean time they have served their purpose and shown that ouabain has no significant acute pro-inflammatory effects in mice. It is possible that ouabain is modulating IL-1β release but that this is not of importance in this model due to the preponderance of effects from other cytokines. It is more likely, however, that murine ouabain resistance has proven sufficient to prevent effects from even the highest doses of ouabain used. Any long-term effects possible were overwhelmed by the severity of disease in the antigen induced arthritis model and more subtle effects that could have been observed in the zymosan model were lost due to the short duration of the model. A more suitable murine model might involve a long-term ouabain pre-dosing schedule followed by the use of low dose intra-articular zymosan to induce disease.

These results certainly do not rule out the possibility of ouabain having pro-inflammatory effects but indicate that a different animal model is required. The α_1 isozyme of Na^+/K^+ATPase is not present in animals such as monkey, dog and rabbit so perhaps these species could provide a more suitable model of inflammation. As investigations at Knoll could no longer be continued this question mark will have to remain.

CHAPTER 4

Effect of Na^+/K^+ATPase Modulation on Human Mononuclear cell Cytokine Production

4.1: INTRODUCTION

The regulation of the balance of cytokine production in health and disease is an area of intensive research. Despite considerable effort many questions remain as to how physiological processes such as inflammation are normally controlled and how they often become unrestrained in diseases such as rheumatoid arthritis (RA).

Within the immune system inhibition of the Na^+/K^+-ATPase with the cardiac glycoside ouabain has been shown to suppress T and B lymphocyte activation in vitro (218) and to potently induce IL-1β production by human monocytes (219). Recent studies by Matsumori et al (231,431) have also shown that various drugs used to treat heart failure, including the cardiac glycosides, modulate cytokine release from various cell types. Inhibition of membrane Na^+/K^+-ATPase results in accumulation of intracellular sodium and depletion of intracellular potassium. In human monocytes Walev et al (1995) have demonstrated that intracellular potassium has regulatory effects on IL-1β processing. Using agents such as staphylococcal α-toxin and gramicidin, both of which selectively permeabilise plasma membranes for monovalent cations, the ionophores nigericin and valinomycin, and the Na^+/K^+-ATPase inhibitor ouabain they demonstrated that K^+ depletion triggered processing of proIL-1β (235).

Whether cardiac glycosides affect monocyte cytokine production via depletion of intracellular potassium of elevation of intracellular sodium remains to be determined. The important feature that is critical to these studies is that the mechanism by which the cardiac glycosides modulate intracellular cation levels is through the inhibition of the key membrane enzyme Na^+/K^+-ATPase.

In rheumatoid arthritis, erythrocyte membranes have been shown to possess markedly suppressed Na^+/K^+-ATPase activity (412). Preliminary work carried out recently in this laboratory has complemented these data by showing decreased Na^+/K^+-ATPase activity in rheumatoid mononuclear cell membranes (243). In normal PBMCs inhibition of the Na^+/K^+-ATPase with ouabain has been shown to mimic the pro-inflammatory cytokine profile seen in RA, with increased production of IL-1β and TNFα and decreased production of IL-6 from monocytes (232). The evidence, of ouabain induced pro-inflammatory cytokine induction and suppression of immuno-regulatory mechanisms, coupled with evidence of decreased Na^+/K^+-ATPase activity

in rheumatoid mononuclear cells provided the impetus for the work presented here. It was felt that further research into the mononuclear cell responses to ouabain would lead to greater insight into the possible biochemical abnormalities that contribute to the pathogenesis of RA. By comparing normal, early rheumatoid and chronic rheumatoid peripheral blood mononuclear cell (PBMC) responses to ouabain it was hoped that the contribution of the Na^+/K^+-ATPase to the regulation of cytokine production in RA would become more apparent. Further to this, it was hoped that more detailed information could be gained on the contribution of intracellular cation levels to the balance within monocytes between pro-inflammatory cytokines such as IL-1β and TNFα and anti-inflammatory cytokines such as IL-1ra.

Chronic rheumatoid patients selected for this study were defined as having sero-positive rheumatoid disease for a period in excess of four years and were preferably not receiving second-line treatments. Patients who had been treated with corticosteroids within the last year were excluded for fear of modulatory effects on cytokine production. The early rheumatoid patients were selected based upon the symptoms of early synovitis and were preferably only receiving non-steroidal anti-inflammatory treatments. Healthy volunteers were used to provide "normal" PBMCs for control responses.

4.2: RESULTS

4.2.1: Effect of Na^+/K^+-ATPase Inhibition on Cytokine Production by PBMCs

Ouabain was used to inhibit the Na^+/K^+-ATPase in human PBMCs and the effect of this modulation on cytokine protein production monitored. Initial experiments were carried out in normal PBMCs to study the dose dependency of ouabain induced IL-1β, TNFα, IL-6 and OSM production. PBMCs were purified from peripheral blood as described in **method 2.2.4** and cultured at a density of 10^6/ml in polypropylene tubes (to avoid adherence activation) for 24hrs in the presence of ouabain (0.1nM to 1μM). As can be seen from **figure 4.1A**, ouabain induced a concentration dependent increase in IL-1β production, with protein production reaching a maximum of 1250pg/ml (mean ±700pg/ml) with a ouabain concentration of 1μM. Similarly,

TNFα was also induced in a concentration dependent manner by ouabain. However, a maximal response of 187pg/ml was seen with 0.1μM ouabain after which TNFα production was seen to be reduced. In contrast, IL-6 and OSM did not appear to be induced by ouabain at all. Production of both these cytokines was very low in all samples, with levels not exceeding 50pg/ml. OSM production appeared to remain at basal levels of approximately 20pg/ml until at a ouabain concentration of 1μM when OSM protein was no longer detected in the supernatants. IL-6 was detected at levels of approximately 5pg/ml in unstimulated PBMCs and ouabain had no effect on this until a slight induction of 20pg/ml was seen at 10nM.

4.2.2: Effect of Ouabain on IL-1β Production by Normal, Early Rheumatoid and Chronic Rheumatoid PBMCs

PBMCs were purified from normal volunteers and patients attending clinics at the Royal National Hospital for Rheumatic Diseases in Bath. Again, cells were incubated in polypropylene tubes, to avoid adherence activation, at a density of 10^6/ml. Before incubation a sample of PBMCs from each patient was assessed for monocyte numbers via CD14 staining using the flow cytometer (FACS). After the supernatants had been harvested for ELISA the cells were tested for viability using trypan blue exclusion under light microscopy or propidium iodide staining analysed by flow cytometry. As can be seen in **figure 4.2,** all three groups responded to ouabain with initial protein induction being seen at 10nM ouabain. However, the concentration response curves to stimulation by ouabain were quite different in the three groups. The normal and early rheumatoid groups gave similar "bell-shaped" concentration response curves with levels of IL-1β production initially being induced and then dropping off. The normal group gave a concentration dependent response with a maximum of 321pg/ml IL-1β being produced with 1μM ouabain. Increased concentrations of ouabain then lead to reduced levels of IL-1β production. The early rheumatoid group gave a concentration dependent response with a maximum of 371pg/ml IL-1β being produced with 0.1μM ouabain. Ouabain concentrations above 0.1μM then gave a decreased level of IL-1β production. In contrast, the chronic rheumatoid group displayed a concentration dependent induction of IL-1β across the whole range of ouabain concentrations studied. The chronic rheumatoid group was

less sensitive to ouabain with comparable levels of IL-1β being produced with ten-fold higher concentrations of ouabain when compared to the normals. These data suggest that IL-1β production was most sensitive to ouabain induction in the early rheumatoid group followed by the normal group and then the chronic rheumatoid group. Maximal levels of IL-1β produced by the three groups were not significantly different but they occurred with different concentrations of ouabain. The early rheumatoid group produced significantly more IL-1β in response to 0.1μM than the normal group ($P<0.05$).

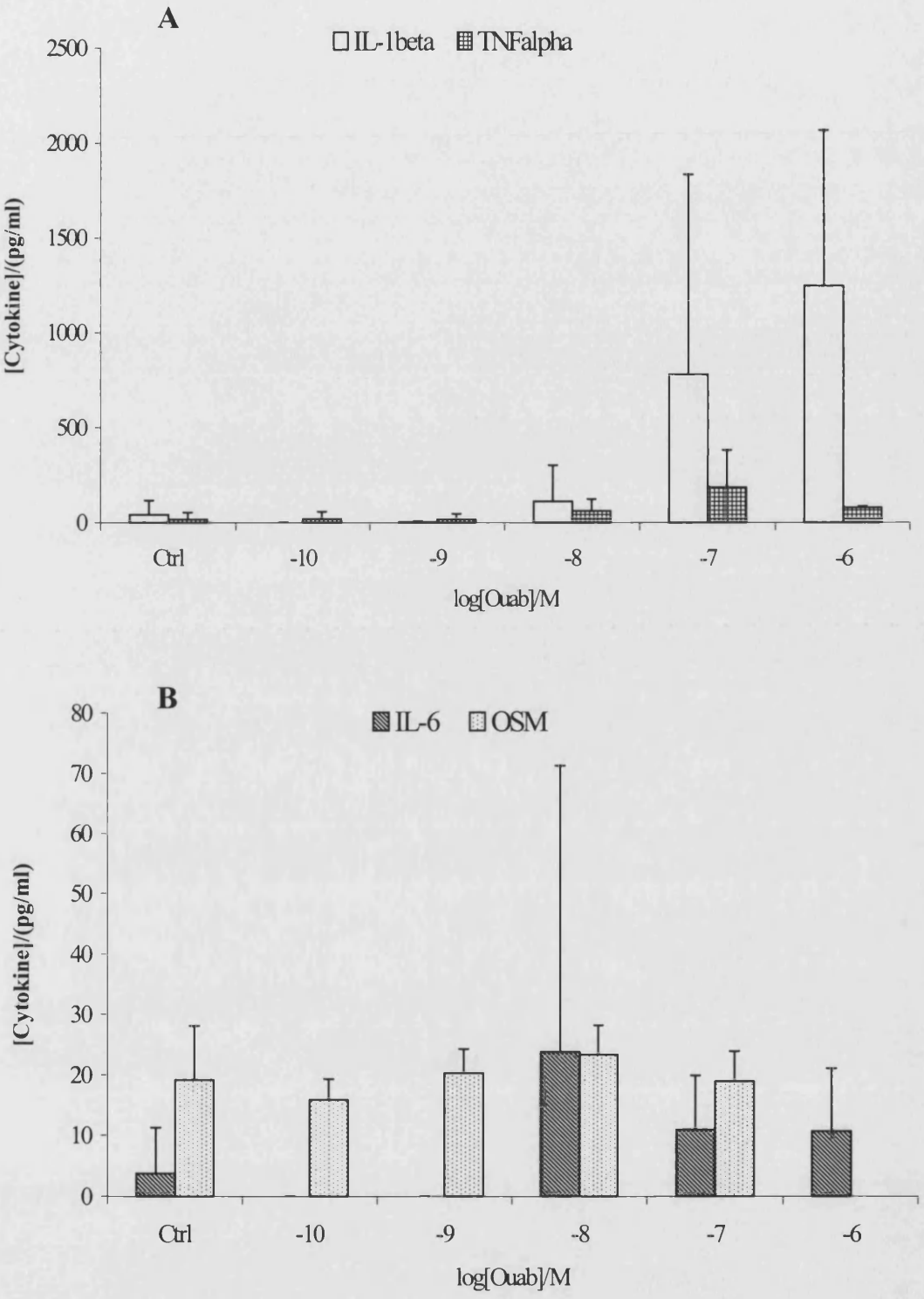


Figure 4.1: Effect of Ouabain on Cytokine Release from Normal PBMCs.
Panel A: Shows effect of Ouabain on basal release of IL-1β and TNFα.
Panel B: Shows effect of Ouabain on basal release of IL-6 and OSM.
Cells were purified from healthy volunteers and cultured with ouabain in polypropylene tubes. Cytokine release was determined after 24 hours (n=4±STDEV).

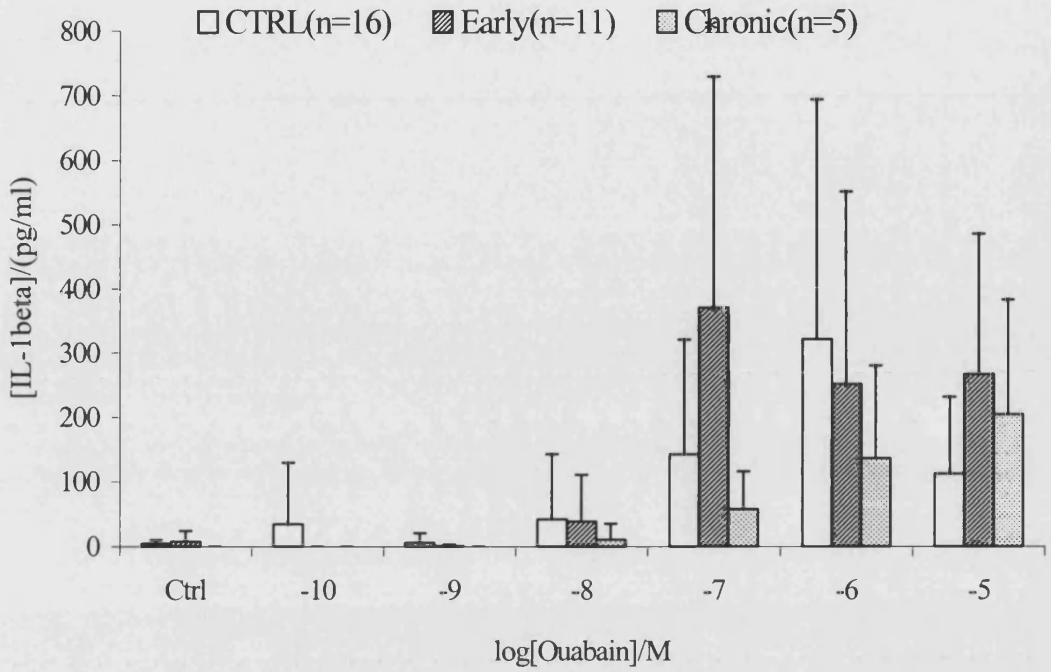


Figure 4.2: Ouabain induced IL-1β from human PBMCs.

Data represents a comparison of normal, early rheumatoid and chronic rheumatoid PBMCs in their response to ouabain. Each point represents an average of all patients in that group ±STDEV, with each protein determination being carried out in duplicate (*=P<0.05).

Patient	% of PBMCs CD14 +ve	STDEV
Control (n=16)	14.99	7.59
Chronic RA (n=5)	17.36	5.65
Early RA (n=11)	13.65	8.94

Table 4.1: Monocyte population of peripheral blood mononuclear cells defined by CD14 staining using flow cytometry.

4.2.3: Effect of LPS on IL-1β Production From PBMCs

The responses to ouabain seen so far in PBMCs complement the evidence that decreased Na^+/K^+-ATPase activity could contribute to the pro-inflammatory cytokine profile in rheumatoid arthritis. Inhibition of the Na^+/K^+-ATPase with ouabain gave a dose dependent increase in IL-1β production and chronic rheumatoid patients appeared to be less responsive to ouabain, perhaps indicating reduced Na^+/K^+-ATPase expression or activity in their PBMCs. One observation with these data, however, was that the levels of IL-1β produced in these experiments was very low when compared with reports from Foey (1997) (232). They were in accord, however, with reports from Newton (1990) (219) who used ouabain (0.1μM) alone to stimulate a maximal release of 700pg/ml IL-1β. This decreased response to ouabain could be the result of numerous factors including different culture medium and foetal calf serum (FCS), different culture conditions (i.e. variations in plastics) or different cell purification techniques. However, it was noted that not only were the response to ouabain reduced in this study but the basal release of IL-1β in the control groups was also very low. Consequently it can be assumed that it is not hypo-responsiveness to ouabain in the studies presented so far but rather general conditions of decreased mononuclear cell activation. In fact, when comparing the results previously presented by Foey in our laboratory to similar studies in the literature, the levels of IL-1β production that he recorded are similar to those reported by Walev (1995) (235) who measured the effect of ouabain on LPS stimulated monocytes. It is possible that changes in culture medium in our laboratory and perhaps lower mitogenicity of the recent FCS used caused the decreased monocyte responsiveness that is recorded here. To test this theory experiments were set up to define ouabain modulation of PBMC responses to the classical IL-1β inducer, bacterial lipopolysaccharide (LPS).

PBMCs were prepared as described previously and the effect of LPS on IL-1β production measured after a 24hr incubation. **Figure 4.3** shows a concentration response curve for LPS from *Salmonella Typhimurium* in normal and rheumatoid PBMCs. Again, control basal levels of IL-1β production were very low, indicating clean, resting culture conditions. With LPS, however, there was a marked induction of IL-1β production with as little as 10ng/ml LPS. The response to LPS reached a plateau at 1μg/ml, with a maximum mean value of 3874pg/ml IL-1β being produced

by normal PBMCs. The response of rheumatoid PBMCs was similar to normal PBMCs but with slightly higher levels of IL-1β being produced. The difference between the two groups was not significant.

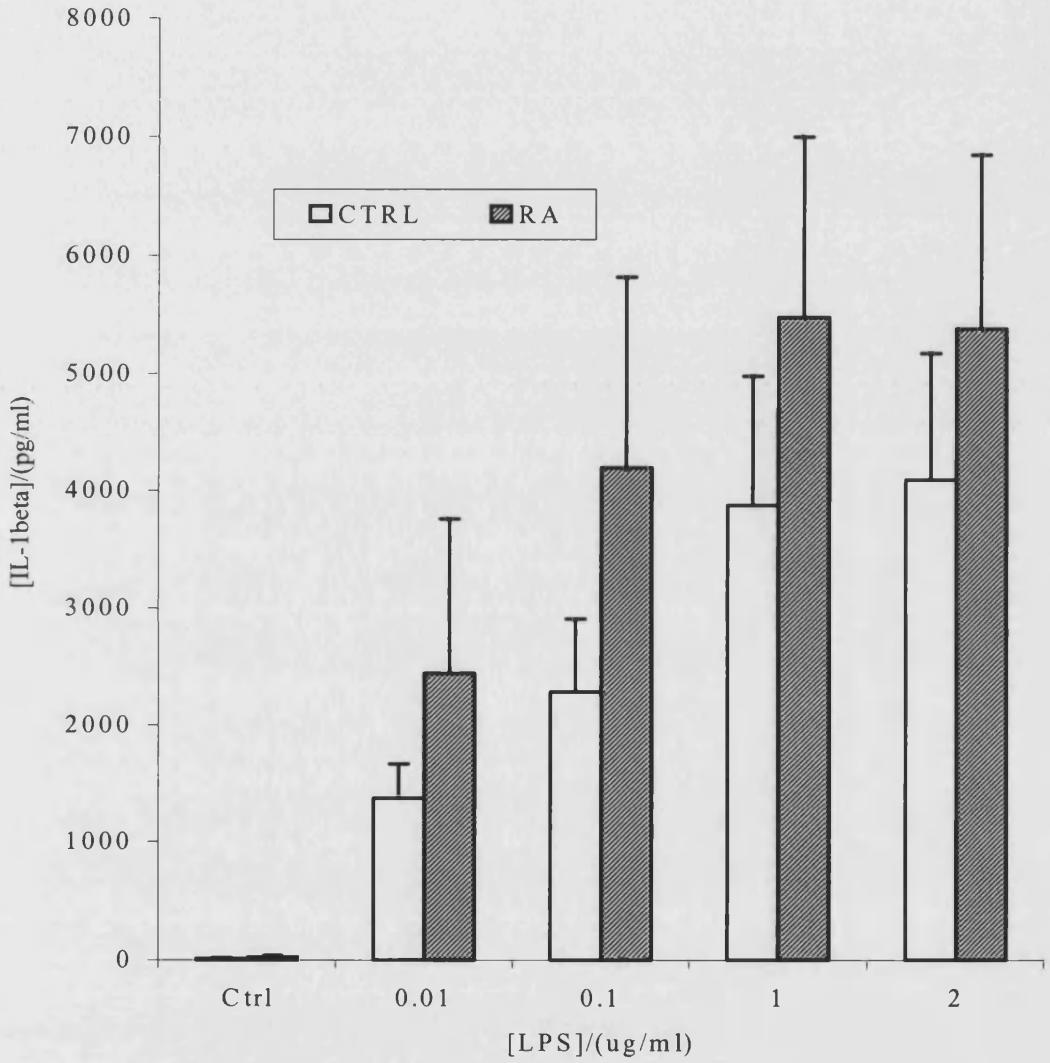


Figure 4.3: Effect of LPS on IL-1β production from human PBMCs.
Data shows response of rheumatoid (n=7) and normal (n=9) PBMCs (± STDEV) cultured for 24 hrs in the presence of various concentrations of LPS (Sal. Typh).

4.2.4: Effect of Ouabain on LPS Induced PBMC Cytokines

LPS was used to prime PBMCs in order to allow the study of the effects of ouabain on activated mononuclear cells. A concentration of 10ng/ml LPS was used as the previous study showed this to give a sub-maximal induction of IL-1β (**figure 4.3**). Culture conditions were repeated as before with effects of ouabain being monitored after a 24hr incubation. LPS and ouabain were added to the samples simultaneously at time zero, with controls receiving only LPS.

Figure 4.4A shows the IL-1β response to ouabain, after LPS activation, and clearly displays the greatly elevated production of IL-1β in comparison to ouabain alone (**figure 4.1**). Control levels of IL-1β production were similar to those seen in **figure 4.3** with 10ng/ml LPS inducing 1156pg/ml IL-1β. A small increase in IL-1β production was seen with 0.1nM and 1nM ouabain inducing 1697pg/ml and 2441pg/ml respectively. With 10nM ouabain, however, a significant increase in IL-1β production was seen ($P<0.05$) with levels reaching 7778pg/ml. On increasing the ouabain concentration to 0.1μM another elevation to 19206pg/ml IL-1β production was observed but further increases in the ouabain concentration lead to a drop in IL-1β production. The production of IL-1β induced by LPS was clearly sensitive to modulation by low levels of ouabain but it was not until ouabain concentrations reached between 10nM and 0.1μM that dramatic synergy was observed.

The same supernatants from the IL-1β experiment above were also tested for TNFα, IL-6 and OSM protein levels. **Figure 4.4B** shows the TNFα response recorded for the effects of ouabain on LPS stimulation. The control response to 10ng/ml LPS was greatly elevated above control basal levels, with a mean level of 2652pg/ml TNFα being produced compared to a basal of approximately 5pg/ml in controls. Similarly to IL-1β, TNFα production was also sensitive to low levels of ouabain with 0.1nM and 1nM ouabain inducing 2866pg/ml and 3228pg/ml TNFα respectively. With TNFα production, however, the synergy between ouabain and LPS was not statistically significant and was not as dramatic as that seen with IL-1β production. Maximal induction of TNFα was observed with 10nM ouabain + LPS inducing 4646pg/ml. Higher concentrations of ouabain reduced TNFα production to levels below those seen with LPS stimulation alone but this inhibition was not statistically significant.

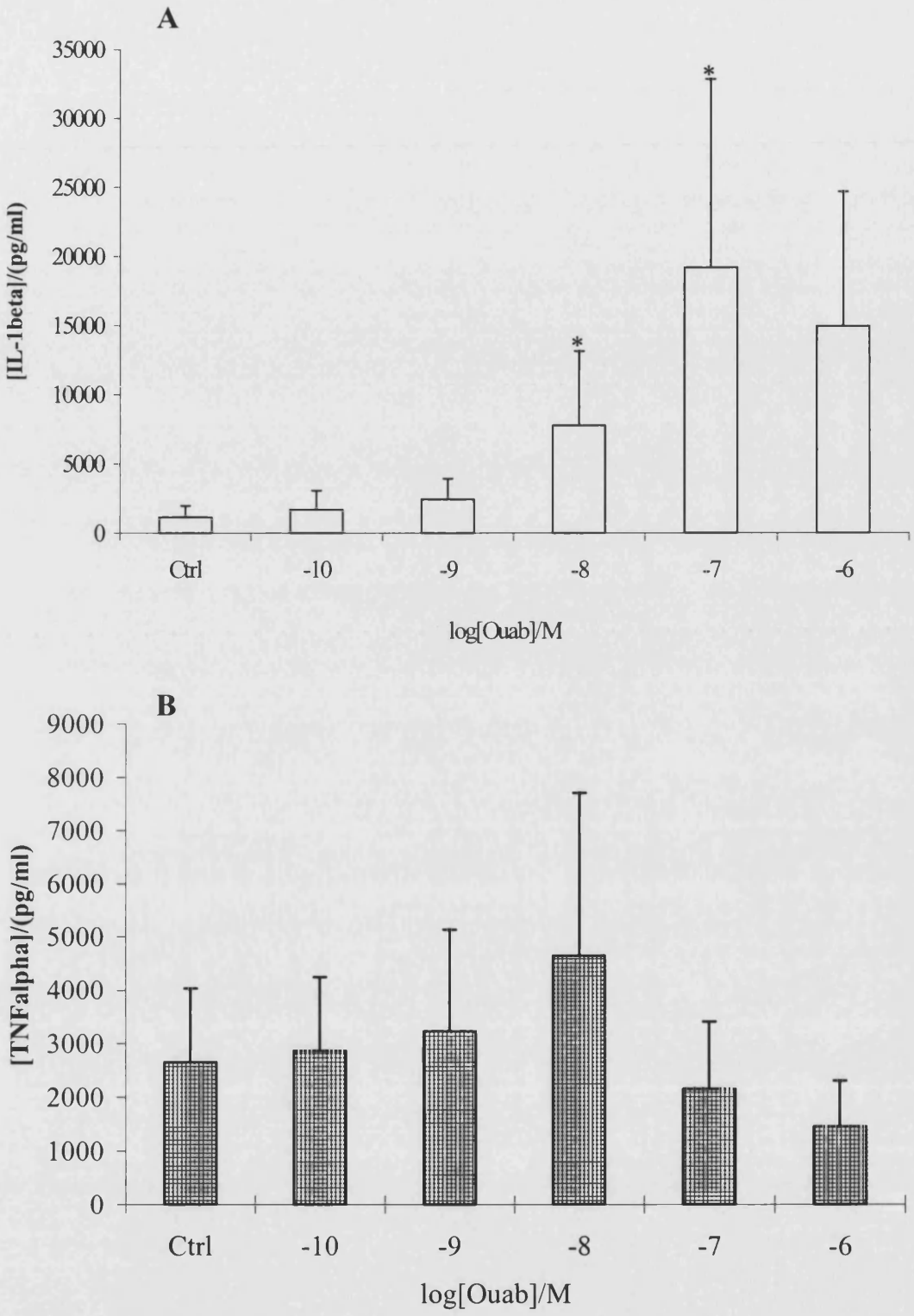


Figure 4.4: Effect of Ouabain on LPS (10ng/ml) Stimulated Cytokine Release from human PBMCs.

Panel A: Shows effect of Ouabain on LPS induced release of IL-1β.

Panel B: Shows effect of Ouabain on LPS induced release of TNFα.

Cells were purified from healthy volunteers and cultured with LPS ± ouabain in polypropylene tubes. Cytokine release was determined after 24 hours (n=4±STDEV, *= $P<0.05$).

Interestingly, the effect of ouabain on LPS induced IL-6 production followed a similar trend to that observed with TNFα. As can be seen from **figure 4.5A**, LPS alone induced 4394pg/ml IL-6 compared to a control value of approximately 5pg/ml. This LPS response was sensitive to low concentrations of ouabain but in contrast to TNFα a maximal induction was seen with 1nM ouabain. At 1nM ouabain a mean IL-6 protein level of 7066pg/ml was observed and although this was 75% greater than that released from control cells due to low experimental numbers this did not prove to be statistically significant. Further elevations in ouabain concentration resulted in levels of IL-6 dropping below control LPS stimulated levels. At 1μM, ouabain reduced the level of IL-6 produced to about 25% of that seen in controls ($P<0.05$).

Of all the cytokines studied in this model OSM was the only one that did not appear to be induced by ouabain. As can be seen from **figure 4.5B**, OSM was induced by LPS, with 10ng/ml LPS stimulating the production of 134pg/ml OSM. This level of OSM production is quite low but significantly greater than that seen in basal control conditions (**figure 4.1**). When ouabain is added to the LPS stimulation, however, there was a concentration dependent inhibition of OSM production, with levels dropping to 29pg/ml with 0.1μM ouabain. At concentrations of 0.1μM ouabain significantly inhibited LPS induced OSM production ($P<0.05$).

4.2.5: Effect of Ouabain on LPS Induced IL-1β From Normal, Early Rheumatoid and Chronic Rheumatoid PBMCs

In this experiment marked synergy was seen in all groups between LPS and ouabain induced IL-1β as it was in the previous study. In contrast to the effect of ouabain on basal release of IL-1β, however, there was no significant difference between any of the three groups. There was a slight variation in the amounts of IL-1β produced by the PBMCs from the different patient groups but this did not extend to significant differences in response to ouabain. As can be seen in **figure 4.6**, maximal IL-1β induction was seen in all groups with 0.1μM ouabain, with normal cells producing 19206pg/ml.

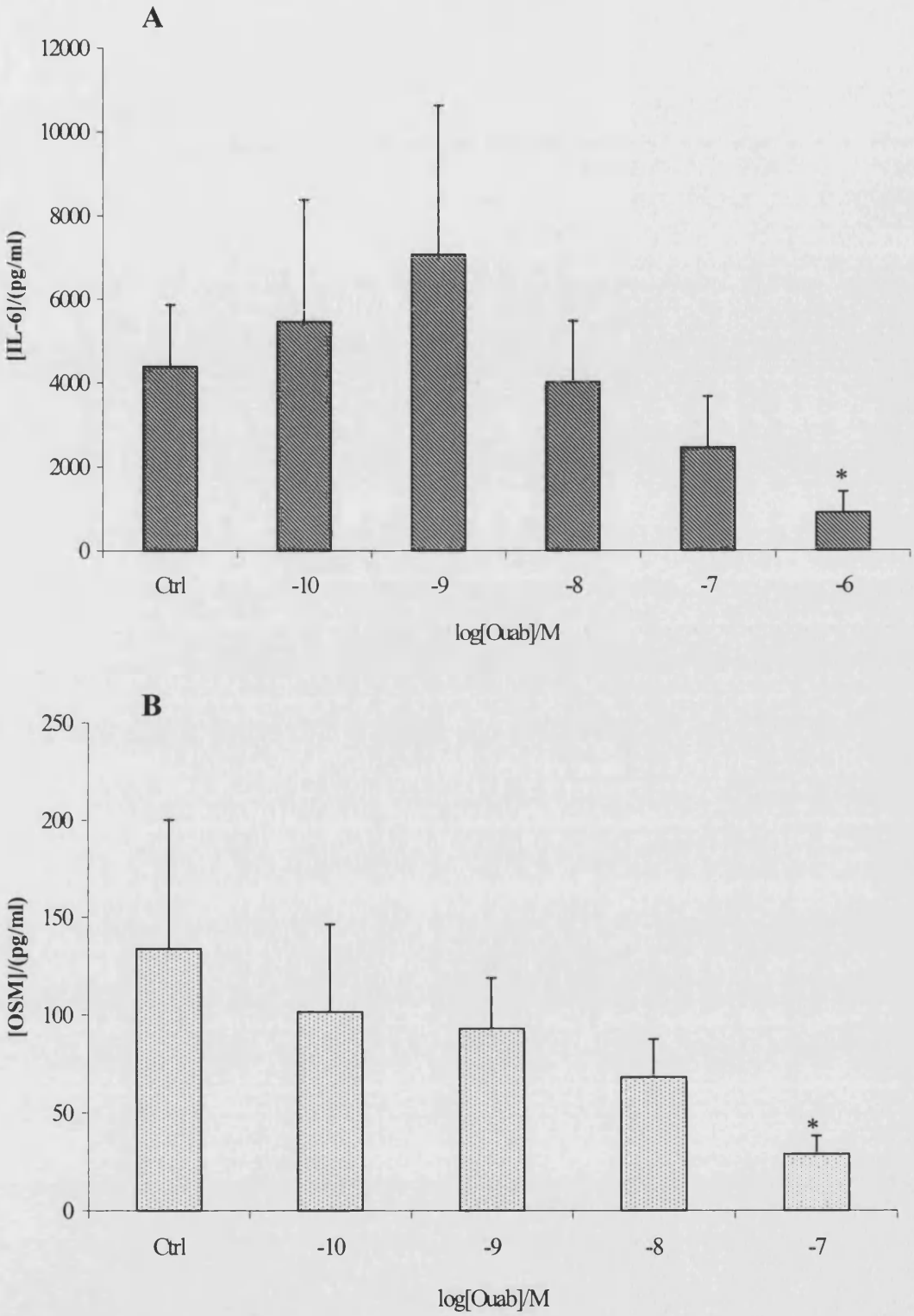


Figure 4.5: Effect of Ouabain on LPS (10ng/ml) Stimulated Cytokine Release from human PBMCs.
Panel A: Shows effect of Ouabain on LPS induced release of IL-6.
Panel B: Shows effect of Ouabain on LPS induced release of OSM.
Cells were purified from healthy volunteers and cultured with LPS ± ouabain in polypropylene tubes. Cytokine release was determined after 24 hours (n=4±STDEV, *= $P<0.05$).

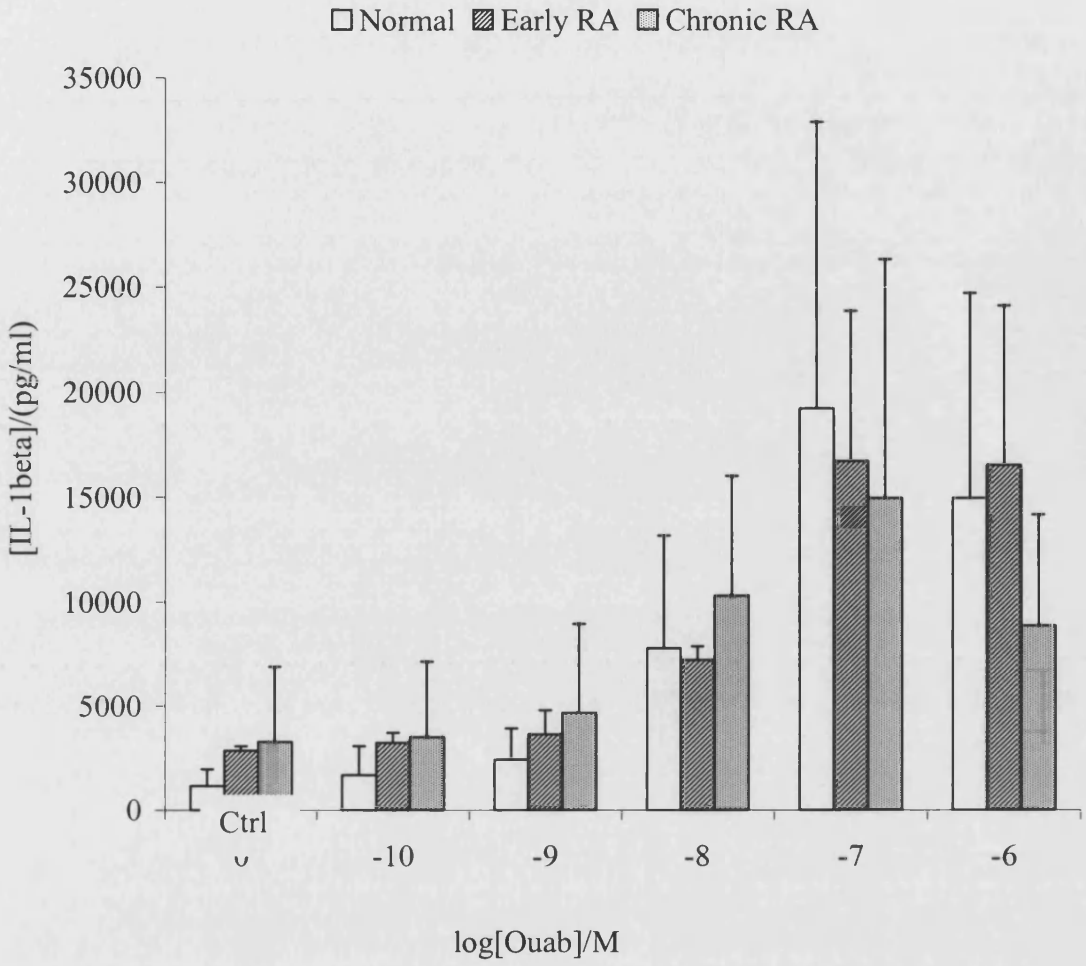


Figure 4.6: Effect of Na^+/K^+-ATPase Inhibition on LPS Induced IL-1β Production from Normal and Rheumatoid PBMCs.
PBMCs were purified from the blood of early RA/synovitis patients, chronic RA patients and healthy volunteers. Cells were simultaneously stimulated with LPS (10ng/ml) and various concentrations of ouabain. Cytokine levels were determined in culture supernatants after 24 hours.
Normal n=16, Early RA n=11, Chronic RA n=5 (± STDEV).

4.2.6: Effect of Ouabain on the Time-course of Cytokine Production Induced by LPS in Normal PBMCs

4.2.6.1: IL-1β

From the previous studies that investigated the concentration dependent effects of ouabain on basal and LPS induced cytokines from PBMCs, it is apparent that ouabain at different concentrations has quite different effects on the various cytokines analysed. Although generally stimulatory at lower concentrations, it was found that at higher concentrations ouabain synergised with LPS to give a dramatic induction of IL-1β and a modest induction of TNFα but tended to inhibit the production of IL-6 and OSM. In the following experiments PBMCs were prepared and incubated as described before but supernatants were harvested every hour for eight hours after stimulation and then at 24 hours after stimulation. All samples were stimulated with 10ng/ml LPS but only 10nM and 100nM concentrations of ouabain were used. After harvesting the supernatants, the cells were lysed to release the intracellular cytokine contents and these too were analysed by ELISA. Cells were lysed in 200μl of lysis buffer so that the concentration of cytokines was comparable to those in the 200μl of culture supernatant. As PBMCs were suspended at $1x10^6$/ml, cytokine determinations in pg/ml represented the cytokine production in pg/10^6 cells.

Figure 4.7 shows the IL-1β response of normal PBMCs. Panel A shows the released cytokine protein measured in the supernatants and panel B shows the cytokine content of the cell lysates. At time zero there is no IL-1β released in to the supernatant or being stored intracellularly, confirming that these cells are in a resting state. With 10ng/ml LPS stimulation mature 17kD IL-1β protein is detectable within one hour. The level of IL-1β detectable in the cell lysates rises rapidly over the next six hours until a maximal level of 100ng/ml is present by seven hours after stimulation. Ouabain at both concentrations studied had no effect on intracellular IL-1β content until at the five-hour time point when a noticeable drop was seen with 100nM ouabain. This effect remains over the rest of the 24hour time-course, with 10nM ouabain having little effect on intracellular IL-1β levels and 100nM ouabain causing a significant drop. By the 24 hour time-point intracellular IL-1β levels in the control (LPS) group and the 10nM ouabain (+LPS) group are comparable at 55ng/ml but in the 100nM ouabain group levels are undetectable ($P<0.05$). The IL-1β content

of the supernatants complements the intracellular cytokine data. In this figure there is little IL-1β detectable in any of the supernatants until at the three-hour time-point when a mean value of 456pg/ml is detectable in just the 100nM ouabain samples. At the four-hour time point IL-1β becomes detectable in the LPS controls and the LPS+10nM ouabain group with levels around 450pg/ml. In the 100nM ouabain group, however, the IL-1β level rises to 5ng/ml at four hours and then climbs rapidly until a maximum of 50ng/ml IL-1β is detectable at eight hours. In the control LPS group and the LPS+10nM ouabain group released IL-1β levels do not exceed 5ng/ml and plateau between six and twenty-four hours.

4.2.6.2: IL-1ra

To complement the pro-inflammatory IL-1β data in these experiments the level of IL-1ra in the cell lysates and supernatants was also analysed. From these data, shown in **figure 4.8**, it can be seen that IL-1ra is regulated in a very different way to IL-1β in response to LPS. Whereas IL-1β production is rapidly induced by LPS and found to be elevated intracellularly within one to two hours and secreted within three to four hours IL-1ra remains at very low levels for many hours. In these experiments, intracellular IL-1ra remained at the 200-300pg/ml level for the first eight hours of culture and it was not until the twenty-four hour time-point that a significant induction was seen. At twenty-four hours after stimulation intracellular IL-1ra levels reached 12613pg/ml in the control LPS stimulated group. This LPS induction of IL-1ra was inhibited slightly by 10nM ouabain, with levels measured at 8251pg/ml and totally blocked with 100nM ouabain ($P<0.05$). In the complementary supernatant data IL-1ra production was again low for the first eight hours of culture. Levels of IL-1ra were initially undetectable and gradually climbed to 850pg/ml in the control LPS group. Again, it was not until the twenty-four hour time point that significant levels of IL-1ra were secreted, with control levels reaching 4179pg/ml. Ouabain had no effect on LPS induced IL-1ra over the first seven hours of culture. After eight hours of culture the 100nM ouabain group showed a reduced level of 250pg/ml IL-1ra, compared to control LPS induced levels of 853pg/ml. At the twenty-four hour time-point 10nM ouabain had a slight inhibitory effect on LPS induced IL-1ra production but 100nM ouabain almost blocked the response completely, with levels

remaining at 450pg/ml. The ability of ouabain to inhibit LPS induced IL-1ra secretion was marked but unfortunately with this low experimental number was not statistically significant.

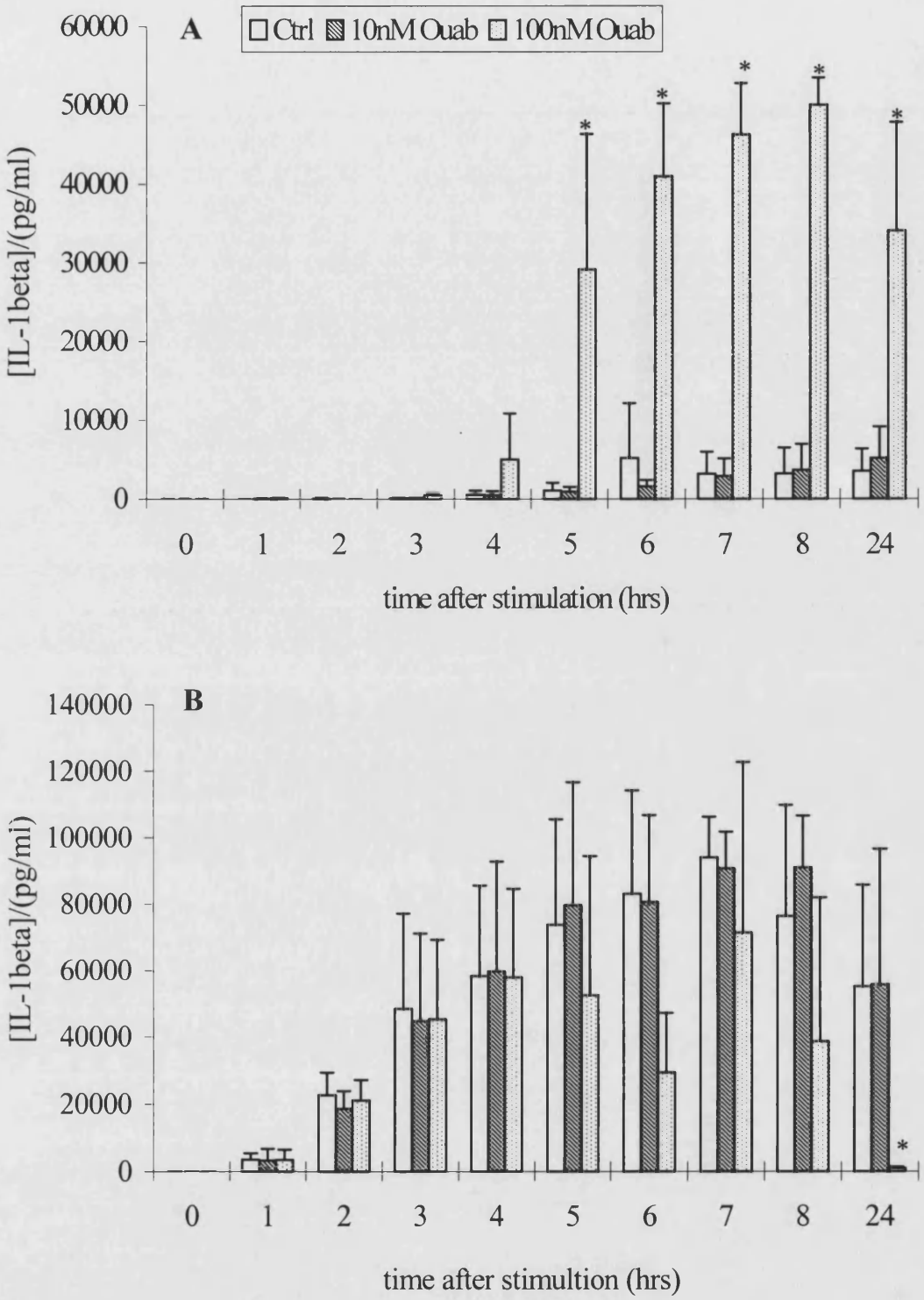


Figure 4.7: Effect of Ouabain on Time-course of LPS Induced IL-1β Production in Healthy PBMCs.

Panel A: Shows time-course for LPS (10ng/ml) induced IL-1β release from PBMCs cultured with or without Ouabain at concentrations of 10nM and 100nM (n=3±STDEV, *= P<0.05).

Panel B: Shows time-course for LPS (10ng/ml) induced IL-1β content of PBMC lysates cultured with or without Ouabain at concentrations of 10nM and 100nM (n=3±STDEV, *= P<0.05).

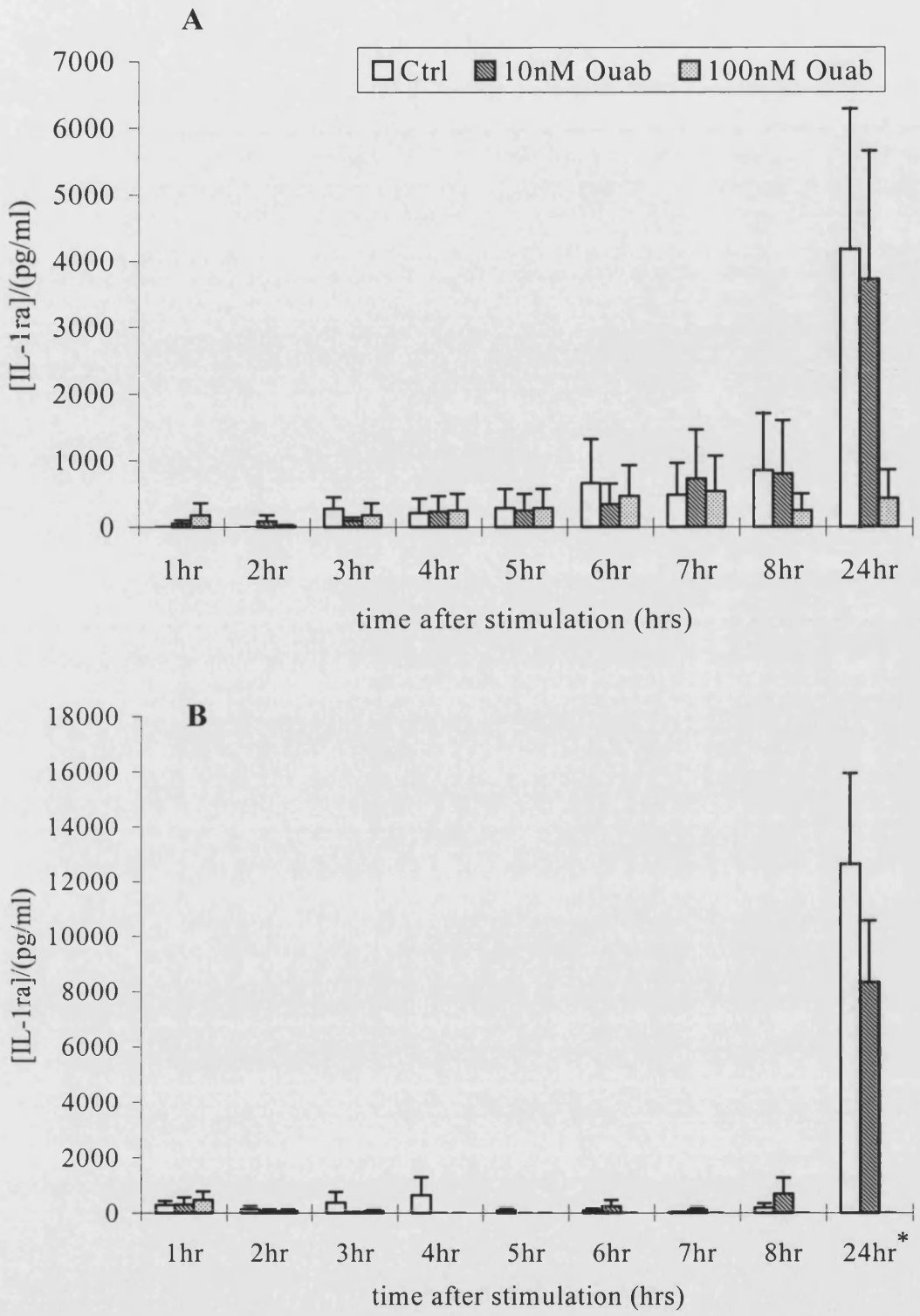


Figure 4.8: Effect of Ouabain on Time-course of LPS Induced IL-1ra Production in Healthy PBMCs.

Panel A: Shows time-course for LPS (10ng/ml) induced IL-1ra release from PBMCs cultured with or without Ouabain at concentrations of 10nM and 100nM (n=3±STDEV).

Panel B: Shows time-course for LPS (10ng/ml) induced IL-1ra content of PBMC lysates cultured with or without Ouabain at concentrations of 10nM and 100nM (n=3±STDEV, *= $P<0.05$).

4.2.6.3: TNFα

The effect of ouabain on LPS induced TNFα release and intracellular TNFα levels is shown in **figure 4.9**. In agreement with the concentration response curve to ouabain, displayed in **figure 4.3**, the twenty four hour time point in this experiment showed that LPS induced TNFα release, that 10nM ouabain enhanced this stimulation and that 100nM ouabain suppressed it. Over the twenty four-hour time-course of this study, however, very little intracellular TNFα was measured in the cell lysates and no obvious effect was seen with either of the ouabain groups. All three groups, control LPS, 10nM ouabain+LPS and 100nM ouabain+LPS showed resting levels of approximately 5pg/ml TNFα at time zero. This level increased to a maximum of 150pg/ml by the five-hour time-point and dropped steadily back to approximately 25pg/ml by the eight-hour time-point. There were tendencies over the time-course for the 10nM ouabain group to display higher levels of TNFα but these were very erratic, statistically insignificant and concerned perhaps insignificantly low levels of protein. In contrast, however, secreted TNFα rapidly reached high concentrations with protein levels being clearly detectable, after just one hour of incubation, at 230pg/ml. LPS induced a steady increase in secreted TNFα over the twenty four hour time-course, reaching a maximum level of 4024pg/ml. The inclusion of 10nM ouabain had no effect on LPS induced TNFα until the last time-point at which it increased the secreted protein level to 5361pg/ml. In contrast, however, from the five-hour time-point 100nM ouabain suppressed the effects of LPS on secreted TNFα production ($P<0.05$) with levels reaching a maximum of 1850pg/ml and remaining constant over the duration of the experiment.

4.2.6.4: IL-6

From the ouabain concentration response curve shown in **figure 4.5** it can be seen that both 10nM and 100nM ouabain had suppressive effects on LPS induced IL-6. Consequently these doses may not have been the best with which to challenge PBMCs for this time-course study. For completion of this data set, however, the IL-6 contents of both supernatants and cell lysates were analysed and displayed in **figure 4.10**.

LPS induced secretion of IL-6 within two to three hours of stimulation. The level of IL-6 released into the supernatants increased steadily until a maximum of 1800pg/ml was reached at the eight-hour time-point. At no point in the twenty-four hour time-course did 10nM ouabain have any effect on LPS induced IL-6 release. In contrast, 100nM ouabain slightly inhibited LPS induced IL-6 production in the four to eight hour samples. However, by the twenty-four hour time-point IL-6 secretion was equivalent in all three groups, at levels of approximately 8000pg/ml.

Intracellular accumulation of IL-6 was rapid after stimulation with LPS. By the two-hour time-point IL-6 levels had increased from a basal production of 5pg/ml to 600pg/ml. The accumulation of IL-6 increased rapidly until a maximum of 2963pg/ml was reached at the four-hour time point. Up until the four-hour time-point neither of the ouabain concentrations had any effect on LPS induced intracellular IL-6. From the four hour to the eight-hour point IL-6 accumulation diminished rapidly in all groups studied, especially the 100nM ouabain group. By the twenty-four hour time-point IL-6 levels were equivalent in all groups at about 300pg/ml.

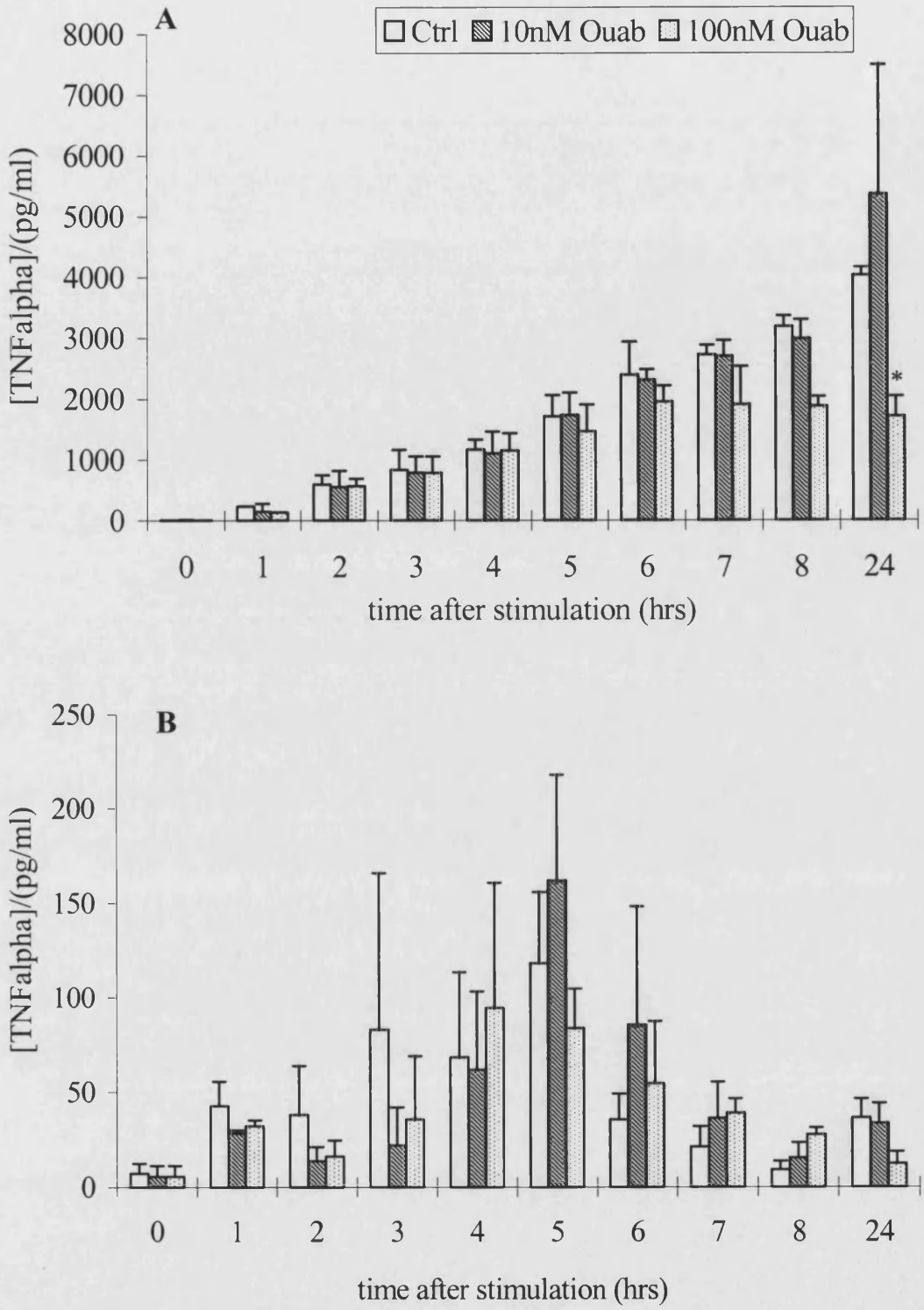


Figure 4.9: Effect of Ouabain on Time-course of LPS Induced TNFα in Healthy PBMCs.

Panel A: Shows time-course for LPS (10ng/ml) induced TNFα release from PBMCs cultured with or without ouabain at concentrations of 10nM and 100nM (n=3±STDEV, *= P<0.05).

Panel B: Shows time-course for LPS (10ng/ml) induced TNFα content of PBMC lysates cultured with or without ouabain at concentrations of 10nM and 100nM (n=3±STDEV).

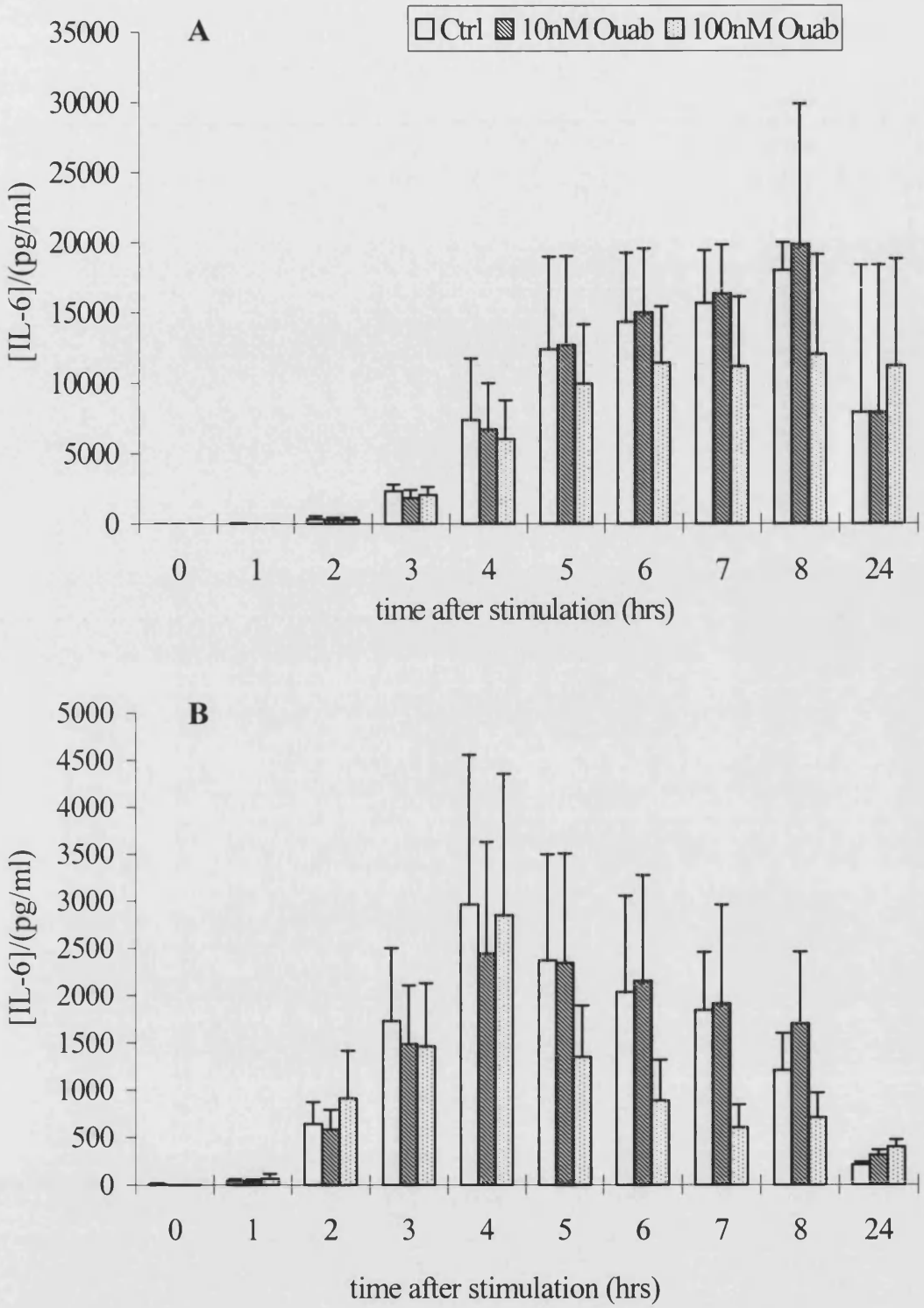


Figure 4.10: Effect of Ouabain on Time-course of LPS Induced IL-6 Production by Healthy PBMCs.

Panel A: Shows time-course for LPS (10ng/ml) induced IL-6 release from PBMCs cultured with or without ouabain at concentrations of 10nM and 100nM (n=3±STDEV).

Panel B: Shows time-course for LPS (10ng/ml) induced IL-6 content of PBMC lysates cultured with or without ouabain at concentrations of 10nM and 100nM (n=3±STDEV).

4.3: SUMMARY

The work detailed in this chapter sought to compare the effects of ouabain on the production of monocyte derived cytokines from PBMCs of rheumatoid patients and healthy controls. The hypothesis of decreased Na^+/K^+-ATPase activity in rheumatoid mononuclear cell membranes was tested via the measurement of functional responses to ouabain in vitro. Ouabain is a specific Na^+/K^+-ATPase inhibitor and thus when used to treat PBMCs in vitro can mimic the decreased Na^+/K^+-ATPase activity suggested in rheumatoid mononuclear cells in vivo. Consequently, the experiments carried out offered the answers to two questions. Firstly, what effect does Na^+/K^+-ATPase inhibition have on the cytokine profile produced by normal PBMCs? Secondly, do the PBMCs from rheumatoid patients display altered sensitivity to the effects of ouabain in vitro?

The results displayed in this chapter show that ouabain has potent modulatory effects on mononuclear cell cytokine production. When used to stimulate resting PBMCs ouabain induced a concentration dependent increase in IL-1β and TNFα release but had little effect on IL-6 and OSM release. Cytokine levels induced by ouabain, however, were not as high as was expected when compared to some similar studies in the literature. Maximum IL-1β levels of 1250pg/ml were induced by 1μM ouabain and maximum TNFα levels of 187pg/ml were induced by 0.1μM ouabain. Production of all four cytokines studied was low but this did, however, offer a very clean system in which to compare the responses of different rheumatoid patients to healthy controls. As IL-6 and OSM release over a twenty-four hour culture were minimally induced by ouabain, rheumatoid and healthy PBMCs were compared for their IL-1β response to ouabain. Chronic rheumatoid patients selected for this study were defined as having sero-positive rheumatoid disease for a period in excess of four years and were preferably not receiving second-line treatments. Patients who had been treated with corticosteroids within the last year were excluded for fear of modulatory effects on cytokine production. The early rheumatoid patients were selected based upon the symptoms of early synovitis and were only receiving non-steroidal anti-inflammatory treatments. When assessing ouabain induced IL-1β production from PBMCs the patient groups were compared for the amount of protein secreted in response to various concentrations of ouabain. As IL-1β is a monocyte derived cytokine all PBMCs were tested for monocyte numbers via anti-CD14

surface staining using flow cytometry. Monocyte numbers were comparable between the three groups but interestingly the IL-1β production in response to ouabain was quite different. The early rheumatoid group showed the greatest sensitivity to ouabain, producing significantly more IL-1β in response to 0.1μM, followed by the normal group and then the chronic rheumatoid group. The maximum level of IL-1β release was seen with 0.1μM ouabain stimulation in the early rheumatoid group, 1μM ouabain stimulation in the normal group and 10μM ouabain stimulation in the chronic rheumatoid group. These data show that IL-1β secretion from PBMCs is modulated by Na^+/K^+-ATPase inhibition and that the different patient groups differ in their sensitivity to ouabain. This variation in sensitivity to ouabain could be a result of different levels of Na^+/K^+-ATPase expression in the different patient groups, different levels of Na^+/K^+-ATPase activity or perhaps differing affinities for ouabain binding. Alternatively, the signal that is induced by inhibition of the Na^+/K^+-ATPase in PBMCs could regulate cytokine gene transcription or protein processing to varying degrees in the different patient groups.

The observation that ouabain did not affect mononuclear cell production of IL-6 contradicts the work of Foey (1997) (232), who demonstrated concentration dependent inhibition with ouabain stimulation of PBMCs. Similarly, it was unexpected that ouabain would have no effect on OSM production. In fact, when the control cytokine responses to ouabain displayed here were compared to similar studies reported in the literature it was felt that they were due to a particularly quiescent culture system. The levels of IL-1β produced here were in accord with data presented by Newton (1990) (219) but were much lower than those described by Walev (1995) (235) who looked at the effect of ouabain on LPS primed mononuclear cells. Consequently, experiments were carried out to study the effect of ouabain on LPS stimulated PBMCs from the different patient groups.

LPS alone was seen to potently induce IL-1β release from PBMCs in a concentration dependent manner. Similarly, LPS induced TNFα, IL-6 and OSM release. Using 10ng/ml LPS to induce a sub-maximal priming stimulus in healthy PBMCs the effect of ouabain was now observed on the production of IL-1β, TNFα, IL-6 and OSM. Ouabain synergised greatly with LPS to induce the release of IL-1β. Similarly, but to a lesser extent, ouabain also synergised with LPS to induce TNFα and IL-6 release. In contrast, however, ouabain caused a concentration dependent inhibition of OSM

release. The maximum response to ouabain occurred at 0.1μM for IL-1β, 10nM for TNFα and 1nM for IL-6 release. Concentrations of ouabain above these maximal effective levels induced a concentration dependent inhibition of the release of the cytokine in question. From these data it would appear that the effects of ouabain on LPS primed PBMCs involve variable concentration dependent responses. In addition to the signal through CD14 induced by LPS there is a complex imbalance in intracellular cation levels induced by ouabain. It would appear that at low concentrations ouabain favours the induction of IL-6 and TNFα and to a similar extent IL-1β but then at concentrations above 10nM to 0.1μM ouabain induces a marked release of IL-1β and down-regulates TNFα and IL-6 release. Whether the decrease in ouabain induced IL-6 and TNFα is as a direct consequence of the IL-1β release cannot be shown here. It is perhaps most likely that at higher concentrations ouabain is inducing such a shift in cation levels that the cells are undergoing a stressed response. Calcium influxes due to elevated intracellular sodium levels may synergise with LPS induced cytokine production initially but at a certain point may over-load the cells and perhaps even deliver a cytotoxic signal.

Comparing rheumatoid and healthy PBMC responses to ouabain after priming with LPS displayed quite a different cytokine profile to that seen in resting PBMCs. Although LPS induced a comparable IL-1β response in rheumatoid and normal PBMCs the addition of ouabain stimulation had less of a variable effect in the different patient groups. Ouabain synergised greatly with LPS induced IL-1β production in all groups studied but there was no difference between the responses of early rheumatoid, chronic rheumatoid and normal PBMCs. In their resting state, the patient PBMCs displayed quite variable sensitivities to ouabain stimulation but when primed with LPS all were equally sensitive to synergy with low concentrations of ouabain.

The complexities of the concentration dependent ouabain induced cytokine profile from PBMCs suggested that there might be time-dependent relationships between the induciton of IL-1β release and effects on IL-6 and TNFα. To explore the possibilities of feedback regulation in the development of the cytokine profile observed with ouabain a time-course of the effects of ouabain on LPS primed PBMCs was carried out. The results displayed in **figures 4.7 to 4.10** show that the synergistic release of IL-1β induced by LPS and 0.1μM ouabain coincides with the decrease in IL-6

production at about four hours after stimulation. The reduction in TNFα release induced by 0.1μM ouabain occurs a little later, at between five and six hours after stimulation. Interestingly, the marked release of IL-1β induced by the combination of LPS and 0.1μM ouabain was mirrored by a drop in the intracellular concentration of IL-1β. This could either indicate that the IL-1β is being secreted more rapidly than it is being made or that intracellular IL-1β processing is being halted at a certain point after stimulation. The latter is perhaps the most likely as the concentration of IL-1β released drops between eight and twenty-four hours.

It is also interesting to note that although the effects of ouabain on LPS induced TNFα release from PBMCs are not evident during the earlier hours of the time-course it would appear that LPS induced TNFα release precedes that of IL-1β.

The cytokine profile induced by ouabain in these studies is typically pro-inflammatory but the exaggerated IL-1β response in LPS primed PBMCs is by far the most dramatic response observed. To test whether the IL-1β family is particularly involved in the response to ouabain, secreted and intracellular levels of IL-1ra were also measured over the same time-course in LPS primed PBMCs. Unfortunately, it appears that the early time-points in this time-course were irrelevant as released and intracellular IL-1ra remained very low until between eight and twenty-four hours. At this point LPS induced a marked elevation in released and intracellular IL-1ra. Interestingly, however, in complete contrast to IL-1β responses, ouabain almost totally abolished the production of IL-1ra both intracellularly ($P<0.05$) and secreted ($n=3$; $P=0.1$).

From these data it would appear that altering the concentration of intracellular cations differentially regulates the production of pro- and anti-inflammatory cytokines. The ability of ouabain to inhibit basal IL-1ra release was not measured but in LPS stimulated studies ouabain significantly favoured the production of IL-1β over that of IL-1ra. If oxidative damage of the Na^+/K^+-ATPase in mononuclear cell membranes in RA occurs the resulting imbalance in intracellular cation levels may well add to the pro-inflammatory. These studies may highlight a possible cycle of events where activation of monocytes infiltrating the inflamed synovium may perpetuate an inflammatory reaction.

CHAPTER 5

Ouabain Induced Death of Monocytes

5.1: INTRODUCTION

The Na^+/K^+-ATPase is an important membrane enzyme that plays a significant role in control of cellular volume and the cationic homeostasis of sodium and potassium. Inhibition of the Na^+/K^+-ATPase with ouabain results in dramatic effects on cationic homeostasis where the electrogenic flux of two potassium ions inwards and three sodium ions outwards is prevented. Consequently, a transient rise in intracellular sodium levels is observed. When a cell has its intracellular sodium concentration artificially raised, it may react using existing cation exchange mechanisms to maintain the sodium ionic homeostasis. It has been described by Blaustein et al (1976) (432) that downstream to rises in intracellular sodium, a concomitant rise in intracellular calcium can be detected, via an increase in the activity of the Na^+/Ca^{2+} exchanger.

Ouabain has been shown to have potent effects on the production of cytokines, especially IL-1β, from PBMCs. Preliminary trypan blue exclusion assays carried out initially in this study showed ouabain to be relatively non-toxic at concentrations less than 10μM. Suspicions of cytotoxicity arose, however, when more detailed studies using propidium iodide staining and flow cytometry were employed. Data indicated a more sinister action of ouabain in the induction of monocyte cytokine expression.

Due to the role of the Na^+/K^+-ATPase in the regulation of cell volume, inhibition with ouabain could cause swelling and necrosis of sensitive cell types. Conversely, it is possible that specific death signals are initiated through alterations in intracellular sodium or potassium concentrations or even via reflex effects on calcium levels within the cell. Apoptosis has been shown to involve a cascade of intracellular proteases, collectively known as caspases (433-435). The IL-1β converting enzyme (ICE), alternatively known as caspase-1, was the first such protein identified on the basis of its sequence homology to the pro-apoptotic *Caenorhabditis elegans* gene product, ced-3 (126). At least ten members of the caspase family have been identified to date (435). Each is capable not only of degrading key cytostructural and reparative proteins but also of activating other caspase family members by cleaving the pro forms of the enzymes at aspartic acid residues. ICE, however, is not only involved in apoptosis, it is also critical in the production of mature IL-1β from the cleavage of pro-IL-1 at an aspartic acid residue. This relationship between IL-1β, ICE and

apoptosis suggested possibilities that if the induction of IL-1β seen with ouabain in the previous chapter was linked with mononuclear cell death, apoptosis could well be involved. Consequently, this chapter set out to investigate possible toxic effects of ouabain on mononuclear cells. To complement the previous cytokine data it was necessary to fully understand the time-course, ouabain sensitivity and nature of any such cytotoxic reactions.

5.2: RESULTS

The effect of stimulation with ouabain on cell viability was tested in PBMCs purified from the blood of RA patients and healthy controls. Initial experiments tested the effects of ouabain over a twenty-four hour culture period in order to complement the cytokine production data shown at the beginning of chapter 4. The aim of these studies was to show that the effects of ouabain on cytokine production were not due to non-specific cytotoxicity.

5.2.1: Effects of Ouabain on PBMC Viability

PBMCs that had been stimulated with various concentrations of ouabain for twenty-four hours were pelleted by centrifugation and retained after the supernatant had been harvested for cytokine ELISA. The cells were then washed once in PBS, re-suspended in FACS sheath fluid and analysed immediately by flow cytometry. The nuclear stain propidium iodide was added to each tube of cells immediately prior to analysis and dead cells, with permeable membranes, were detectable due to high FL-2 fluorescence. As can be seen in **figure 5.1A** basal cell death in PBMCs cultured for twenty-four hours was consistently around 5%. Ouabain had no effect on cell viability until at a concentration of 0.1μM when a doubling in the number of PI positive cells was seen. Increasing concentrations of ouabain gave increased cell death until a plateau was reached at 10μM ouabain. Cell death rarely exceeded 20-30% of the PBMC population and it was observed to correlate often with CD14 expression of the purified PBMC population.

To investigate the possibility that ouabain was having cytotoxic effects on the monocyte population of the PBMCs initial observations used FACS light scatter

analysis. As the PBMCs were being cultured in polypropylene tubes to prevent adherence-activation induced cytokine production it was possible to retrieve all the cells for FACS analysis. By observing the light scattering properties of the PBMC population it was possible to identify and track any changes in the morphology of the monocytes. Using the FACS, when a cell passes through the laser beam some light is reflected and detected as side scatter (SSC) and some light passes by the cell and is detected as forward scatter (FSC). Consequently the cell population can be assessed for size by the amount of light that is detected as FSC and for membrane morphology by the degree of SSC that is detected. As monocytes are larger and more ruffled than lymphocytes, they are detectable as a population of cells in the upper-right quadrant of the PBMC population. To confirm that light scattering properties were a reliable way to identify the monocyte population experiments were carried out using anti-CD14 stained PBMCs that were stained with a FITC conjugate. By plotting FL-1 fluorescence against either FSC or SSC it was clear that it was the high FSC and high SSC cells that became FL-1 bright upon CD14 staining (data not shown). By observing the FSC and SSC of the PBMC population after twenty-four hour stimulation with ouabain it was now possible to identify which cells were being affected. As ouabain inhibition of the Na^+/K^+-ATPase affects cell volume it might be expected that such changes would be visible on the FSC-SSC FACS plot. As can be seen in **figure 5.1B** the monocyte population is clearly distinct from the lymphocyte population as a separate cloud of cells in the upper-right region (for clarity the monocyte population is circled in the first plot). Upon stimulation with ouabain the monocyte cloud initially becomes more diffuse but at concentrations of 0.1μM or above is seen to disappear completely. Interestingly, the lymphocyte population appears to remain unaffected by ouabain. From these results it appears that the cell death induced by ouabain is restricted to the monocyte population of the PBMCs.

Although the FACS data offered compelling evidence for ouabain induced death of monocytes further studies were carried out to investigate the mechanism of this response. Efforts had to be made to correlate cell death with cytokine release in order to understand how ouabain may be modulating monocyte cytokine production.

5.2.2: Ouabain Induced Death of Monocytes

To confirm that ouabain was specifically affecting the viability of the monocyte population of the PBMCs experiments were carried out to purify monocytes from peripheral blood and to study these directly. In theory, this was to be a simple procedure but in practice it proved to be quite difficult to purify a population of monocytes that were in a suitable resting state for experimentation.

Most classical monocyte purification procedures utilise plastic adherence as the main selection tool, with all other PBMCs being washed away. Unfortunately, however, this leaves the monocytes adhered to culture dishes and obviously unsuitable for FACS analysis. Attempts were made to lift the monocytes after plastic adherence so that they could then be cultured with ouabain in polypropylene tubes. It was found, however, that using cell dissociation buffer (Sigma) or even Trypsin EDTA caused a high degree of cell death in itself and proved unsuitable for these studies. The next approach for monocyte purification utilised centrifugal separation over a density gradient. Percoll concentrations of 50% to 54% were tested but unfortunately these also retained the larger lymphocytes which were of a similar buoyant density to the monocyte population. A second density gradient approach was tested using NycoPrep™ (Nycomed). NycoPrep has a density of 1.068g/ml, which therefore retains the more buoyant cells in a similar manner to Percoll. The difference with NycoPrep, however, is that it has an osmolality of 335mOsm that causes the lymphocytes to shrink, becoming more dense and passing through the gradient. Purified monocytes can then be collected from the plasma/gradient interface. These monocytes were found to be greater than 70% CD14 positive and their viability remained reasonably constant and high in control cultures.

The effect of ouabain on the viability of enriched monocytes was assessed using PI staining on flow cytometry. Cells were cultured with various concentrations of ouabain and assessed for viability after eight, sixteen and twenty-four hours. As can be seen in **figure 5.2A** ouabain caused a concentration and time dependent increase in cell death. Death was only apparent in the 0.1 and 1μM ouabain groups and only significantly noticeable after sixteen hours of culture ($P<0.05$). Although marked, the ability of 0.1μM ouabain to induce death of monocytes was not statistically significant over the twenty-four hour study. Increased experimental numbers would probably prove 0.1μM ouabain to at least be toxic after twenty-four hours.

To complement the data on ouabain induced death of peripheral blood monocytes experiments were also carried out using the monocytic cell line THP-1. The THP-1 line had already been characterised in terms of Na^+/K^+-ATPase activity and thus proved to be a suitable model for studying the effects of ouabain on cell death. Resting THP-1 were cultured for twenty-four hours with various concentrations of ouabain and assessed for viability using PI staining. The results for this experiment are displayed in **figure 5.2B** and show that THP-1 cells are similarly sensitive to ouabain induced death as peripheral blood monocytes. No effect on cell viability is seen with ouabain stimulation until at a concentration of 0.1μM when significant cell death is observed ($P<0.05$). Cell death increases with increasing concentrations of ouabain until a plateau is reached with 10μM ouabain.

The effects of ouabain on PBMC cytokine production occur rapidly, with IL-1β release being measurable within four hours of stimulation (**section 4.2.6.1**). The data presented here, using PI staining to assess viability of purified monocytes, suggests that little toxicity occurs in ouabain treated cells within eight hours of culture. PI staining, however, gives no information on the state of cell activation so attempts were made to study the monocyte population in more detail over a time-course of ouabain stimulation. The time-course of ouabain effects on monocyte morphology were assessed using FSC and SSC analysis on flow cytometry. Healthy PBMCs were cultured with 1μM ouabain and cells harvested for analysis at three, five, six, eight and eighteen hours. A typical FACS trace is shown in **figure 5.4** with the monocyte cloud being clearly distinct from the lymphocyte cloud. With ouabain treatment it can be seen that as early as three hours after stimulation the monocyte cloud appears to increase slightly in FSC and SSC. The monocytes become slightly larger and more distinct from the lymphocyte population, perhaps as a result of increased volume or activation. Five hours after stimulation the monocytes appear to be a more diffuse population but it is not until between six and eight hours that the size of the monocyte population appears to be reduced. By eighteen hours after stimulation the entire monocyte cloud has disappeared.

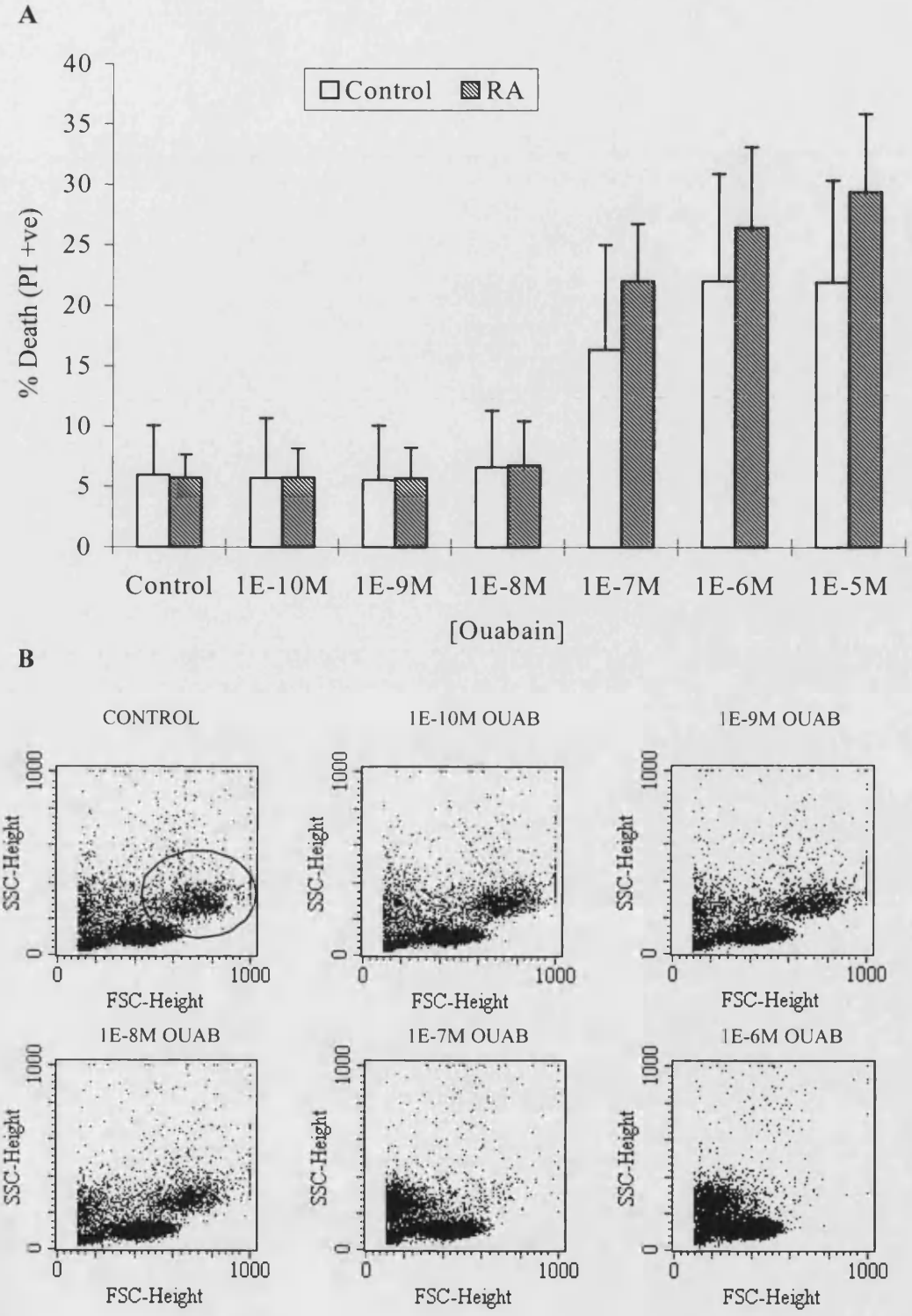


Figure 5.1: Ouabain induced death of PBMCs.

Panel A: Death of PBMCs incubated with various concentrations of ouabain for twenty-four hours. Cell death is measured as an increase in FL-2 fluorescence, due to uptake of the nuclear stain propidium iodide, detected using flow cytometry. (RA, n=11, Control, n=9 ±STDEV).

Panel B: Ouabain concentration response curve in PBMCs incubated for twenty-four hours. The plots display the light scattering characteristics of the cell population and highlight the effect of ouabain on the monocytic cells. The monocytic population can be identified, due to its increased size and more ruffled surface, as the upper right cell cloud (circled in the first plot).

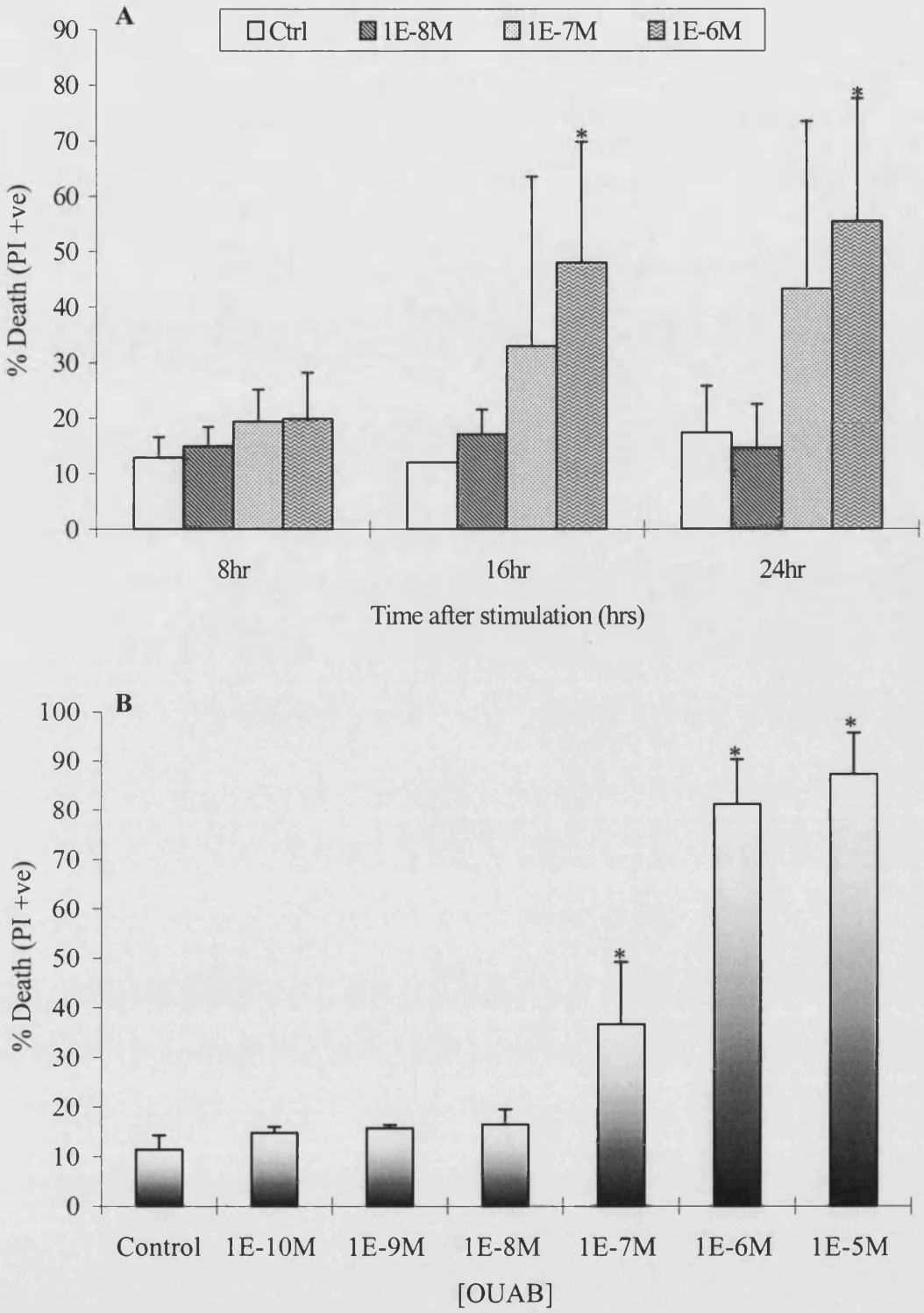


Figure 5.2: Ouabain induced death of monocytes.
Panel A: Ouabain-induced death of purified human blood monocytes after twenty-four hour incubation (n=3±STDEV, *= P<0.05). Death measured as PI staining by flow cytometry.
Panel B: Ouabain-induced death of THP-1 cells after twenty-four hours incubation (n=3±STDEV, *= P<0.05). Death measured as PI staining by flow cytometry.

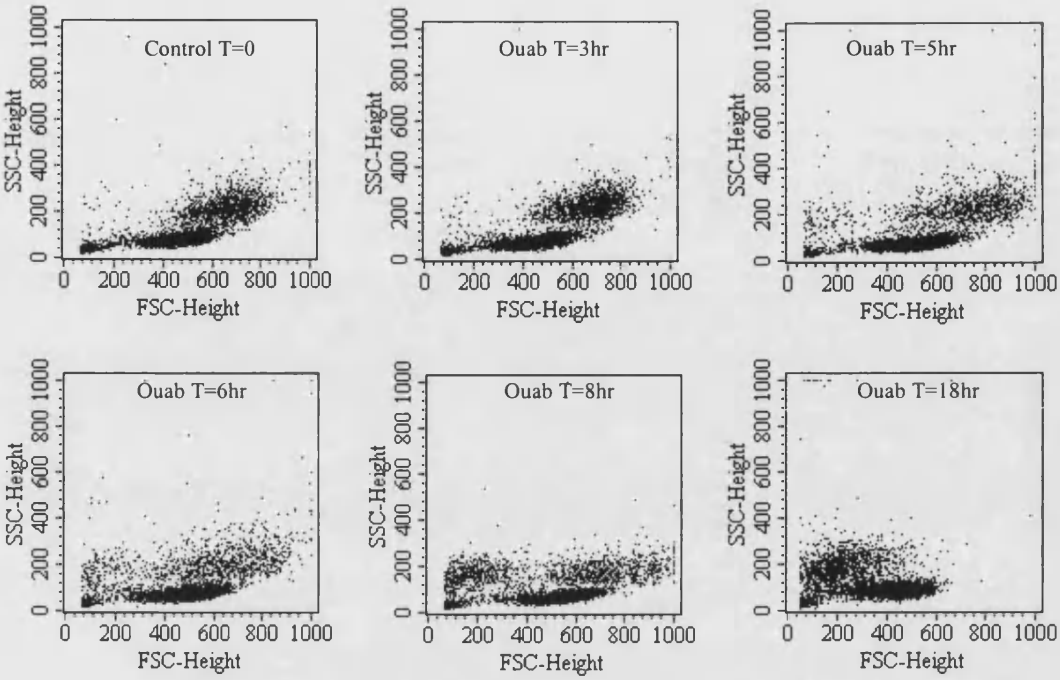


Figure 5.3: Time-course of Ouabain-Induced Death of Monocytes.
PBMCs from normal individuals were treated with 1E-6M ouabain and tested for viability at various times after stimulation. The monocytic population was identified as the upper-right cloud on the FACS scatter plot.

5.2.3: Ouabain Induced Apoptosis of Monocytes

The molecular mechanisms of apoptosis are not fully understood but are known to involve the IL-1β converting enzyme (ICE) family of proteases. In a variety of cell systems ICE family proteases have been shown to be activated during apoptosis (436-439). Also, in similar studies, over-expression of certain proteases caused cells to undergo apoptosis (434,440-446). Inhibitors of the ICE family proteases have been used to block the induction of apoptosis in other cell systems (436,437,447). In this study ouabain has been shown to dramatically induce production of IL-1β and cause significant toxicity in monocytic cells. As ICE is the enzyme responsible for the production of mature IL-1β and may also be involved in apoptosis, experiments were carried out to see if ouabain induction of IL-1β may involve apoptotic pathways.

Annexin V staining of phosphatidyl serine (PS) was used to distinguish between apoptotic and necrotic death of monocytes using flow cytometry. PS has been shown to be externalised during apoptosis (433,448,449) and is one of the identifying features that allows macrophages to recognise and eliminate apoptotic cells. As apoptotic cells externalise PS in the early stages of apoptosis without losing membrane integrity they will not take-up the nuclear stain propidium iodide. When stained with Annexin V–FITC (FL-1) and PI (FL-2) necrotic cells will become both FL-1 and FL-2 bright when analysed by flow cytometry. Under the same conditions apoptotic cells stain FL-1 bright and FL-2 dark.

Purified peripheral blood monocytes and the monocytic cell line THP-1 were used to study the mechanisms of ouabain induced cell death. As the degree of cell death shown via PI staining in **figure 5.2** is only just measurable eight hours after stimulation this time-point was initially used as a starting point for the study of Annexin V binding. Purified peripheral blood monocytes were stimulated with 0.1μM ouabain and harvested at various time points for double staining with PI and Annexin V. Typical results are shown in **figure 5.4** where the FACS plot is split into four quadrants. The lower-left quadrant represents the double-negative cells, i.e. those that are alive. The lower-right quadrant represents the Annexin positive, PI-negative cells, i.e. those that are apoptosing. The upper-left quadrant represents cell debris and the upper-right quadrant represents the double-positive cells, i.e. those that are necrotic or have lost membrane integrity after apoptosis. Over twenty-four hours

of culture the majority of the monocyte population remains healthy, in the lower left quadrant. There is a little background AnnexinV staining and a little necrosis, probably due to the stress of purification and culture. In the ouabain treated group, however, there is a measurable induction of death that displays the clear characteristics of apoptosis. In the eight-hour ouabain treated group there is little difference in the FACS profile when compared to controls. By twelve hours, however, there is a clear increase in both the apoptotic population and the necrotic population. After twenty-four hours of stimulation there are still cells that appear to be undergoing apoptosis and the percentage of necrotic cells has increased slightly. The outcome of these apoptotic cells was not followed further than twenty-four hours.

In an attempt to simplify the study of ouabain induced death the THP-1 cell-line was utilised for further experiments on apoptosis. THP-1 cells are described as being pro-monocytic and undergo differentiation when treated with $1,25(OH)_2$-vitamin D3 (352). Differentiated THP-1 cells express CD14 and in many respects behave like normal peripheral blood monocytes. The effects of ouabain on THP-1 cell death, measured by PI staining, were similar to those seen in blood monocytes so it was hoped that apoptosis responses would be similar. As can be seen in **figure 5.5**, however, it appears that in THP-1 cells ouabain does not induce an apoptotic response. Both resting and differentiated THP-1 cells did not develop an annexin bright population upon stimulation with ouabain. Ouabain was tested from 0.1μM to 10μM over a twenty four hour time-course but all toxic effects induced were detected as necrosis. The live cells cross straight from the double-negative quadrant to the double-positive quadrant. The results displayed in **figure 5.5** are representative of three experiments and detail the early events in the induction of death. Interestingly, THP-1 cells die more rapidly in response to ouabain stimulation than peripheral blood monocytes and it also appears that differentiated THP-1 cells are more sensitive than resting THP-1 cells. At 6.5 hours after ouabain stimulation four-fold more necrotic cells were present in the differentiated THP-1 cells than in the control THP-1 cells.

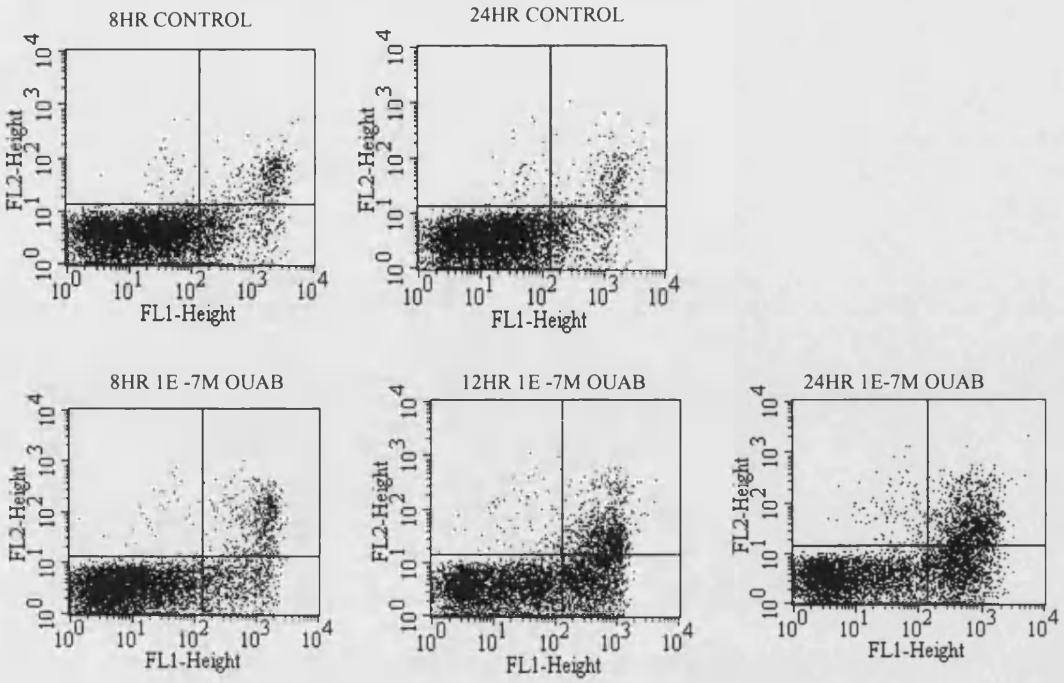


Figure 5.4: Ouabain Induced Apoptosis of Purified Peripheral Blood Monocytes

Purified monocytes were treated with 1E-7M ouabain and then stained with Annexin V to detect apoptosis (FL-1 +ve, lower right). Necrosis was differentiated from apoptosis using propidium iodide to double stain (FL-1 + FL-2 +ve, upper right).

Quadrant	Control		1E-7M Ouabain			
	8 hour	24 hour	8 hour	12 hour	16 hour	24 hour
Upper Left	0.70%	0.67%	0.56%	1.39%	0.86%	1.89%
Upper Right	5.30%	3.20%	7.57%	14.70%	13.60%	18.89%
Lower Left	83.37%	88.40%	81.83%	60.34%	63.91%	56.9%
Lower Right	10.63%	7.73%	10.04%	23.57%	21.63%	22.33%

Table 5.1: Quadrant Statistics for data shown in Figure 5.4.

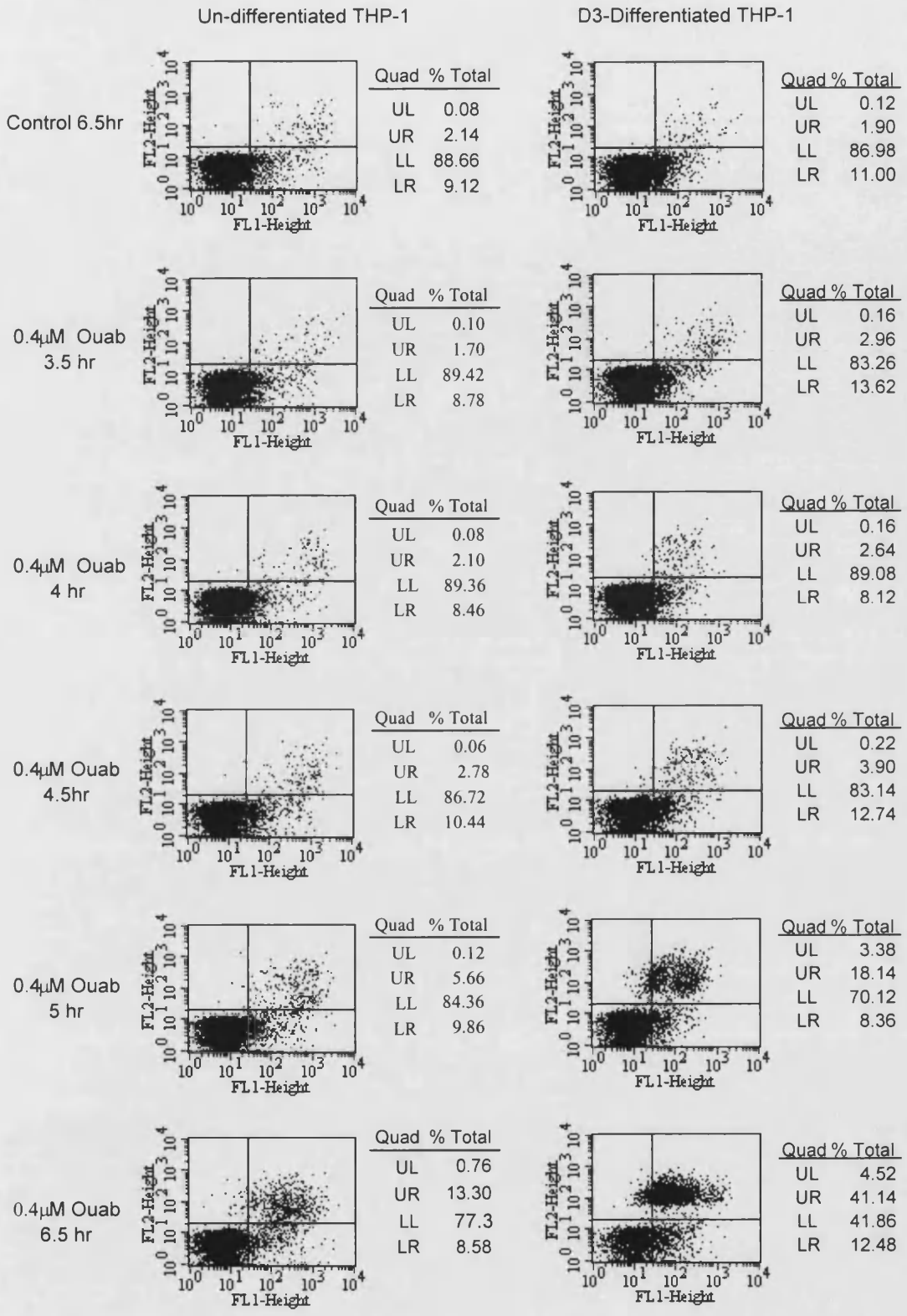


Figure 5.5: Ouabain Induced Death of THP-1 Cells.
D3-differentiated and undifferentiated THP-1 cells were cultured with 0.4μM ouabain for various times. Cell viability was assessed using Annexin V and PI staining. Quadrant definitions: LL = live cells, LR = apoptotic cells, UL = cell debris and UR = necrotic cells.

5.2.4: Effect of LPS on Ouabain Induced Monocyte Death

The effects of ouabain on PBMC cytokine production, especially IL-1β, were enhanced greatly by LPS stimulation. To test whether this may involve cytotoxicity PBMCs were stimulated with both ouabain and LPS and assessed by flow cytometry using scatter analysis. Typical responses, representative of three experiments, are shown in **figure 5.6**. Eight hours after stimulation, cells treated with 10ng/ml LPS did not vary greatly to control cells. The monocyte population of the PBMCs was a little more diffuse than controls, perhaps indicating activation-induced changes in morphology. In this system, at eight hours, ouabain alone had no effect on the monocyte population. The addition of 10ng/ml LPS, however, rendered the monocytes more sensitive to ouabain induced death. LPS + 10nM ouabain had no effect on cell viability but the combination of 0.1μM ouabain and LPS caused marked disruption of the monocyte cloud.

To complement these data purified monocytes were stimulated with ouabain ± 10ng/ml LPS and assessed for death using PI staining. The cells were cultured for twenty-four hours so controls were expected to be relatively insensitive to 10nM ouabain but to die readily when stimulated with 0.1μM ouabain (compare with **figure 5.2A**). The mean data of two experiments are shown in **figure 5.7** and clearly demonstrate that LPS treated monocytes are more sensitive to ouabain induced death.

To test whether LPS was affecting ouabain-induced apoptosis of peripheral blood monocytes or was merely contributing to activation induced necrosis, Annexin V staining was again employed. Purified monocytes were stimulated with ouabain and LPS over a twenty-four hour time-course similar to that displayed in **figure 5.4.** At no point, however, did the addition of LPS contribute to the degree of ouabain induced apoptosis. In fact, as shown in **figure 5.8**, LPS stimulation resulted in a decrease in ouabain induced apoptosis and an increase in the percentage of necrotic cells.

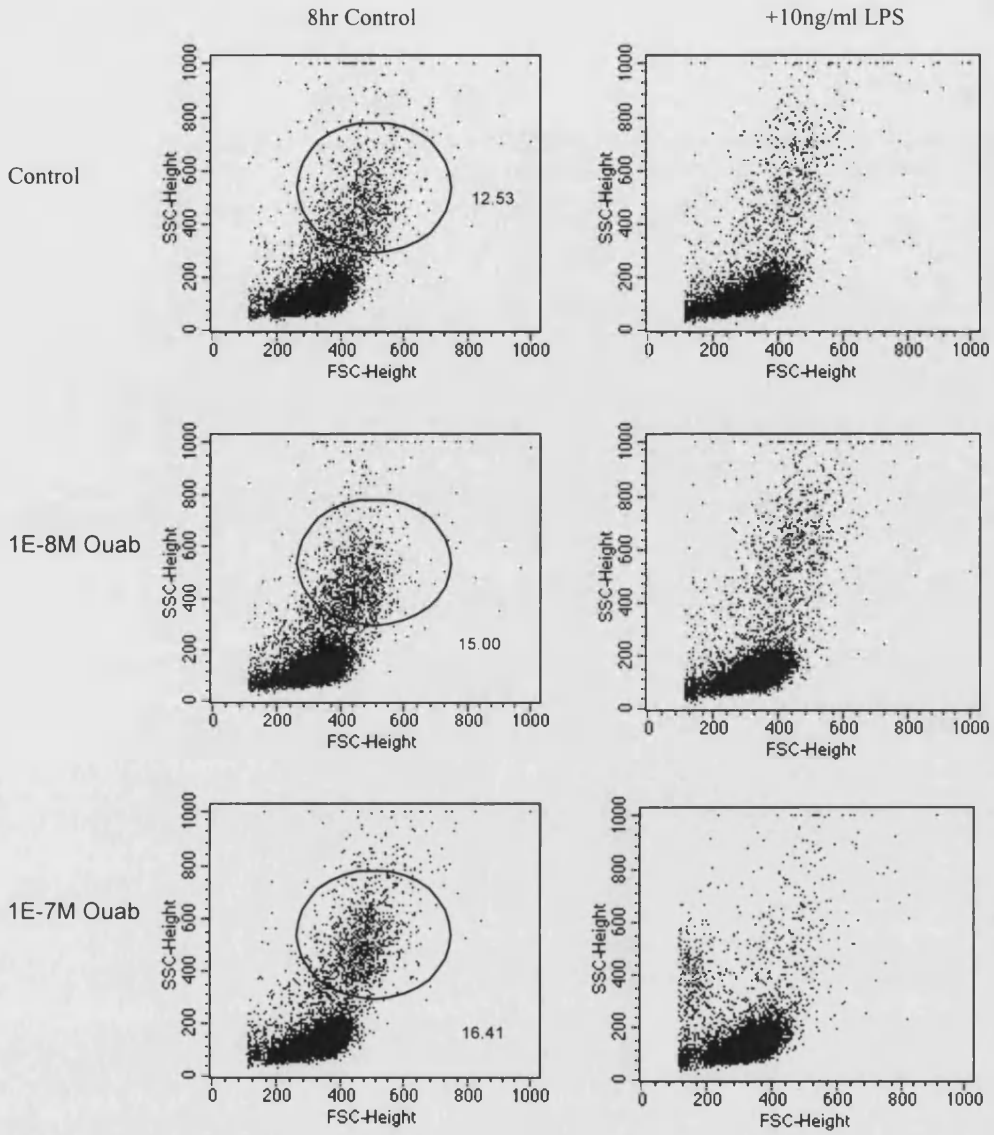


Figure 5.6: Effect of LPS on Ouabain-Induced Monocyte Death.
PBMCs were incubated for twenty-four hours and the viability of the monocyte cloud was visualised from FACS scatter plots. The effect of 10ng/ml LPS was assessed against control and ouabain treated cells.

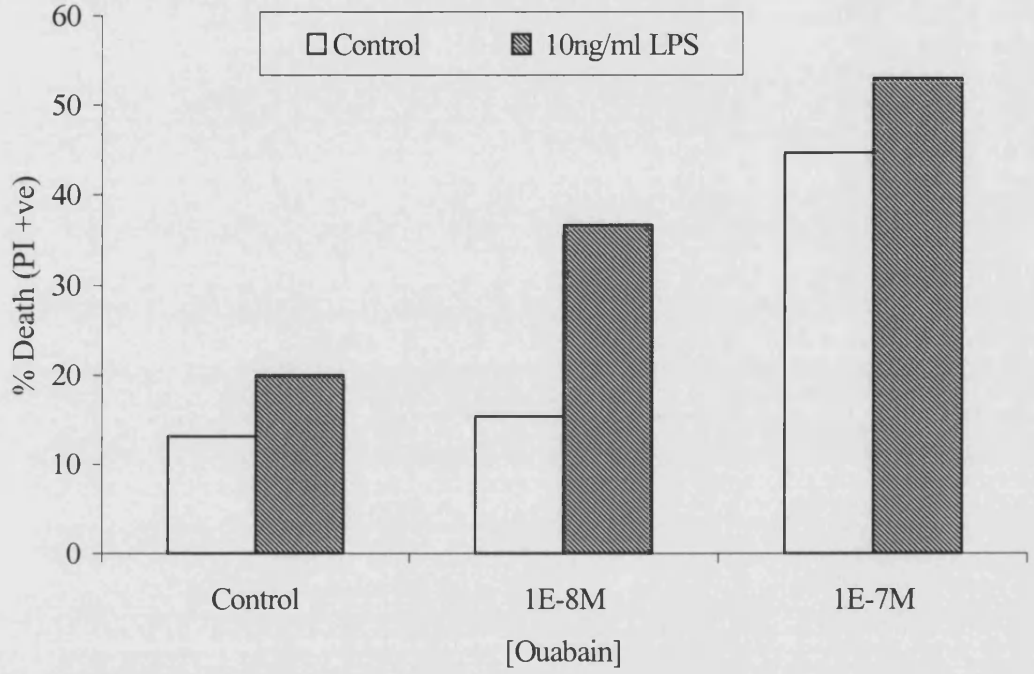
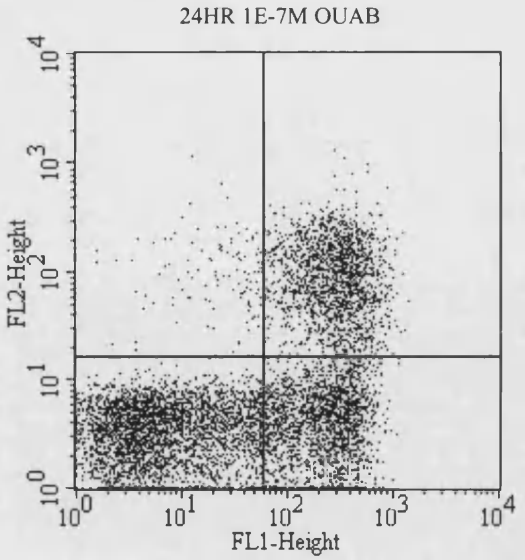
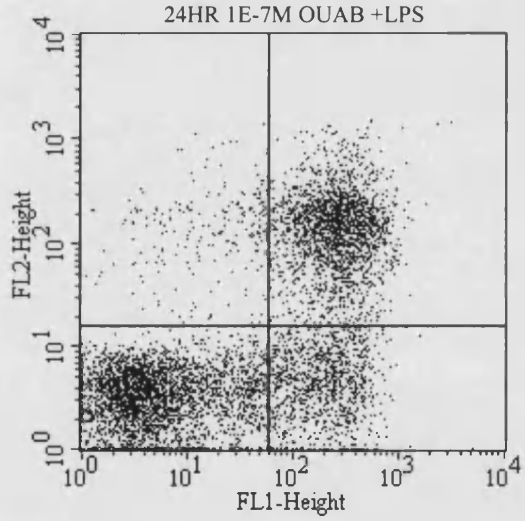


Figure 5.7: Effect of LPS on Ouabain-Induced Death of Enriched Monocytes. Purified human blood monocytes were incubated with various concentrations of ouabain for twenty-four hours ± 10ng/ml LPS. Cell viability was determined using the nuclear-stain propidium iodide (PI). Data represents the mean of two experiments.



Quad	% Total
UL	1.80
UR	25.43
LL	45.99
LR	26.79



Quad	% Total
UL	3.54
UR	33.13
LL	47.63
LR	15.70

Figure 5.8: Effect of LPS on Ouabain-Induced Monocyte Apoptosis.
Purified human blood monocytes were stimulated with 1E-7M ouabain ± 10ng/ml LPS for twenty-four hours. Cell viability was assessed using the combination of Annexin V and propidium iodide staining. Apoptotic cells bind just Annexin V (FL-1 +ve, lower right). Necrotic cells bind both PI and Annexin V (FL-1 + FL-2 +ve, upper right).

5.3: SUMMARY

When measured using propidium iodide (PI) staining assessed by flow cytometry, ouabain concentrations above 0.1μM were seen to cause a low percentage of death in resting PBMCs. There was no mean difference between rheumatoid and healthy samples but it was noticed that the degree of death would often correlate with the number of monocytes observed in the PBMC preparation using anti-CD14 staining. Complementary data recorded using FACS scatter analysis of whole PBMC populations and PI measurements in purified monocyte preparations and the monocytic cell line THP-1 cells showed that ouabain concentrations above 0.1μM induced cell death after eight hours of stimulation. Between three and six hours of stimulation monocyte enlargement could be observed but after this the monocyte population disappeared from the FACS plots.

Based upon the data displayed in chapter four it appears that before death is observed there is a large release of IL-1β. Due to evidence that ICE (Caspase 1) may well play a role in apoptosis, experiments were carried out to try and define whether ouabain may be inducing an apoptotic response in monocytes and that the secretion of IL-1β may be related to over-activation of ICE during apoptosis. Although the results displayed here are by no means conclusive, based upon AnnexinV staining, they do suggest that in purified blood monocytes ouabain induces apoptosis to occur between eight and twenty four hours after stimulation. However, THP-1 cells did not display any signs of apoptosis and were seen to die via necrosis upon stimulation with ouabain.

Ouabain was seen to synergise dramatically with LPS to induce IL-1β release so the effects of LPS on ouabain induced death of monocytes was also measured. LPS promoted ouabain-induced death after eight hours stimulation with 0.1μM ouabain and 10ng/ml LPS. However, death did not occur as early as the production of IL-1β so it is possible that cytokine production or release could be related to a specific death response. It seems unlikely that the cells are simply dying and releasing their contents as the monocyte preparations appear quite healthy according to analysis by flow cytometry for several hours after cytokine production is elevated. The synergy between LPS and ouabain in the induction of IL-1β does not transfer to an apoptotic

effect as if anything LPS appeared to promote necrosis after twenty-four hours of stimulation with 0.1μM ouabain.

From these data it is possible that the synergy between ouabain and LPS in inducing monocyte IL-1β production may well involve an apoptotic event. From the studies with PBMCs in chapter 4 it can be seen that LPS alone induces the secretion of IL-1β and intracellular accumulation of high levels of IL-1β but no measurements were made in relation to intracellular levels of pro-IL-1β. From the evidence presented in this chapter it is possible that ouabain induced apoptosis of peripheral blood monocytes could result from activation of ICE and that the increased production of IL-1β is as a consequence of increased processing of intracellular pro-IL-1β. Experiments using specific ICE inhibitors such as Zvad would help to define the role of ICE in ouabain induced secretion of IL-1β and induction of apoptosis.

In this model it is possible that the outcome of stimulation with ouabain depends upon the substrate availability for ICE. It has been shown that intracellular proIL-1β protects cells against apoptosis via competing with apoptotic substrates for cleavage by ICE (524). In the model described here it is possible that if ouabain induces the activation of ICE the enzyme may favourably process LPS induced proIL-1β until levels start to decline. After which ICE starts to cleave components of the apoptotic cascade. However, ouabain did not induce apoptosis of THP-1 cells but has similar effects on THP-1 production of IL-1β as those seen with LPS stimulated PBMCs (data not shown). Consequently the question arises as to whether ouabain may act at the level of cytokine secretion, that apoptosis occurs via feedback stimulation of monocytes and that THP-1 cells may be insensitive to such an apoptotic mechanism.

At high concentrations of ouabain it is possible that cell necrosis occurs simply due to the high osmotic stress that the cells are under. The time-course of death events demonstrated here, however, supports the data that cytokine secretion occurs first. The fact that ouabain selectively inhibits IL-1ra release whilst markedly inducing IL-1β release further supports the evidence that ouabain selectively modulates cytokine release and that death also occurs via specific processes rather than simple necrosis.

CHAPTER 6

Regulation of monocyte IL-1β and IL-1ra production: T cell/Monocyte Co-culture

6.1: INTRODUCTION

The goal of this chapter was to study the differential regulation of monocyte IL-1β and IL-1ra production and to establish a model for the determination of T cell induced monocyte cytokine release. Such a model could well have fulfilled the data requirements of an entire thesis and as a result what is presented here only really scratches the surface of a fascinating area of monocyte regulation. Due to time constraints some of the data is unfortunately not as strong as it could be and represents what were really pilot studies in the development of the co-culture model. The need to develop this model was hastened by the award of a Basic Science Initiative grant from Pharmacia and Upjohn, the proposal for which was to assess the ability of Sulphasalazine (SPZ) to modulate T cell activation and T cell driven monocyte cytokine production.

By developing a greater understanding of signals that regulate the balance between IL-1β and IL-1ra production from human monocytes it was hoped to develop a model suitable for the study of immuno-modulatory compounds. In this case a model suitable for the study of anti-rheumatic compounds such as SPZ would be equally as useful for the study of compounds such as ouabain. By identifying signals that favour the production of IL-1β or IL-1ra one could establish a model in which the balance of these two cytokines can be studied and compared. It would be possible to compare the balance between IL-1β and IL-1ra production from rheumatoid and normal monocytes in response to control stimuli. Also, using a model monocyte, it would be possible to observe the ability of T cells from rheumatoid patients and controls to induce monocyte responses. After developing such a model it was hoped to be able to study the effects of ouabain against control IL-1β and IL-1ra stimuli. It would be interesting to see whether ouabain could modulate monocyte responses to stimuli which favour the production of IL-1ra as well as synergising with LPS induced IL-1β production.

The co-culture system developed here was based upon that used in the laboratories of J-M Dayer (352), who showed that activated T cells could stimulate THP-1 cytokine production. Using the THP-1 cell line Dayers' group has demonstrated T cell modulation of MMP expression (379), IL-1β production and IL-1ra production

(381). THP-1 cells have also been used to demonstrate differential induction of IL-1β and IL-1ra by Th1 and Th2 T cell clones (397).

One of the aims of this part of the thesis was to compare the ability of normal and rheumatoid T cells to induce cytokines from THP-1 cells and also to look at the response of the autologous monocytes to Jurkat T cells. This meant first having to define T cell activation, in response to various stimuli, in terms of proliferation and cell surface marker expression.

Before THP-1 cells could be used as a model for the study of T cell driven cytokine responses they first had to be characterised in terms of basal cytokine production. As the main focus of this chapter is the balance between IL-1β and IL-1ra production positive control responses for each of these cytokines had to be established. From previous studies presented in this thesis, and extensive reports in the literature, LPS was chosen as the primary candidate for induction of IL-1β. Early studies using THP-1 cells had reported that they are unresponsive to LPS stimulation. More recent reports published by Dayer, however, have demonstrated that after differentiation with 1,25-$(OH)_2$-vitamin D3 THP-1 cells readily produce IL-1β in response to LPS activation. Dayer has also measured IL-1ra responses from THP-1 cells in response to LPS but more classical stimuli that have been reported include immune-complexes and aggregated IgG. For the purpose of this study plastic bound IgG was investigated as a suitable positive control for monocyte IL-1ra induction.

In all experiments using THP-1 cells for cytokine production the cells were cultured at an initial density of $5x10^5$/ml. It was found that, at greater densities, THP-1 cells rapidly over-crowded the well, especially in long time-course experiments. All the following experiments were carried out in 96 well round bottom polystyrene plates (Falcon) with a maximum volume of 250µl per well. As THP-1 cells in resting or differentiated form are non-adherent there was no concern for adherence induced activation. Supernatants were harvested at the end of culture by gentle aspiration after pelleting of the cells at 350g in a Beckman microplate GH3.7 rotor.

6.2: RESULTS

6.2.1: IL-1β but not IL-1ra Production by THP-1 cells is dependent on Vitamin D3 differentiation

THP-1 cells were cultured at a density of $5x10^5$/ml in RPMI-1640 with $1,25(OH)_2$-vitamin D3 (D3). D3 is reported to induce responsiveness to LPS and as CD14 is a monocyte marker and receptor for LPS this study monitored CD14 induction as a marker of THP-1 differentiation. As can be seen from **figure 6.1**, D3 induced a concentration dependent increase in CD14 expression in THP-1 cells. CD14 expression was observed to be increased within twenty-four hours of activation and to have reached a plateau between forty-eight and seventy two hours. For the following experiments D3 differentiation was carried out using a concentration of 10nM D3 cultured with the THP-1 cells for forty eight hours.

When resting THP-1 cells were compared to differentiated THP-1 cells (D3 THP-1) for their responsiveness to LPS and IgG it was found that only LPS responses were D3 dependent. **Figure 6.2** displays the IL-1β response to LPS and the IL-1ra response to IgG. It can be seen that normal THP-1 cells produce no IL-1β under resting conditions and only 163pg/ml after stimulation with 5μg/ml LPS. In contrast, D3 THP-1 produce a large quantity of IL-1β, with 1937pg/ml being released on twenty-four hour stimulation with 5μg/ml LPS. In response to plastic bound IgG, however, both normal and D3 THP-1 produce IL-1ra in equivalent amounts. FACS surface staining data complements these results as it is seen that CD14 is up regulated with D3 differentiation but Fc receptor expression remains constant (**figure 6.1B**).

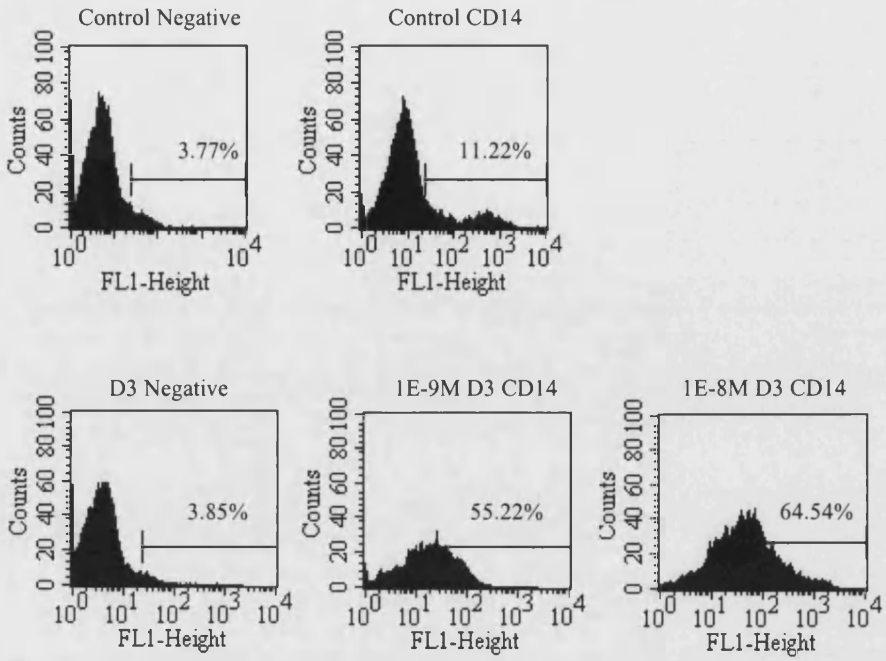


Figure 6.1A: 1,25(OH)$_2$-Vitamin D3 Induced CD14 Expression on THP-1 Cells
Cells were cultured for forty-eight hours in different concentrations of D3 and CD14 induction determined by FACS analysis.

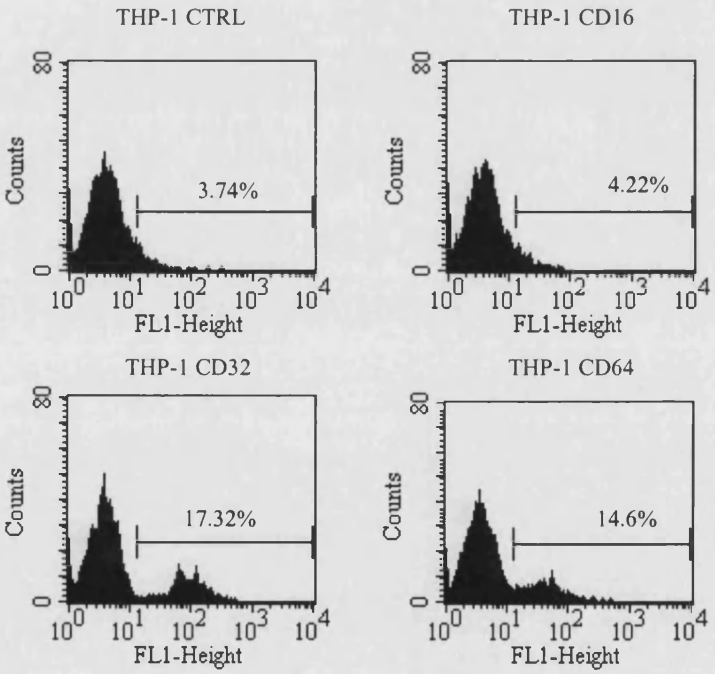


Figure 6.1B: FcR Expression in Normal THP-1 cells.
THP-1 cells from resting cultures were analysed for expression of CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI). D3 differentiated THP-1 cells were found to have identical surface FcγR phenotypes.

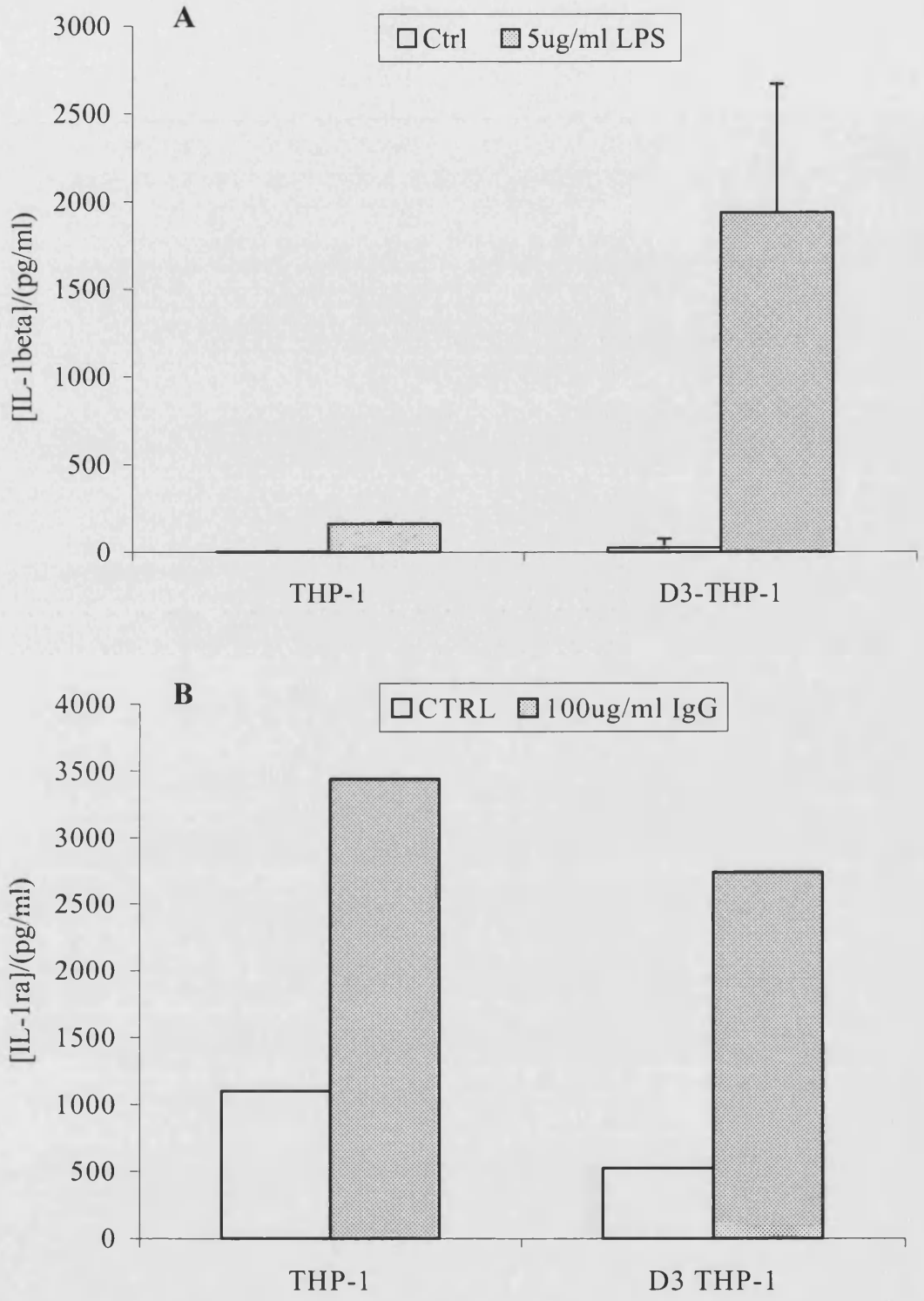


Figure 6.2: Effect of D3-Differentiation on THP-1 Cytokine Production.
Panel A: D3-THP-1 cells were differentiated for 48 hours before being stimulated with 5g/ml LPS for 24 hours. Responses were compared to undifferentiated THP-1 cells (n=3±sem).
Panel B: D3-THP-1 cells were differentiated for 48 hours before being cultured on plastic bound IgG for 48 hours. Responses were compared to undifferentiated THP-1 cells (n=2).

6.2.2: Time-course of IL-1β and IL-1ra production By D3 THP-1 cells

The release of IL-1ra from the LPS and ouabain stimulated PBMCs studied in chapter four was much slower than that of IL-1β. In order to be able to study the balance between IL-1β and IL-1ra production from THP-1 cells suitable time-points for their comparison had to be determined. In the following experiments D3 THP-1 were cultured with varying concentrations of LPS and the IL-1β and IL-1ra protein secretion measured over a seventy-two hour time-course. **Figure 6.3** represents IL-1β release in the top panel and IL-1ra release in the bottom panel. As can be seen in both graphs, stimulation with LPS gave a concentration dependent increase in IL-1β and IL-1ra production. However, IL-1ra production was very low over the first twenty-four hours of culture and increased rapidly over the last forty-eight hours of the time-course. In contrast, IL-1β production did not increase significantly between twenty-four and seventy-two hours.

The data represented in **figure 6.4** also reflects the time-course of IL-1β and IL-1ra production from D3 THP-1 cells. This time, however, it applies to IgG driven cytokine production over a forty-eight hour time-course. The first obvious feature of these data is that plastic-bound IgG is a poor inducer of IL-1β, especially in comparison to its effects on IL-1ra levels. Although IgG driven IL-1β production appears to be more time dependent than LPS driven IL-1β production, the differences between the levels measured at twenty-four and forty-eight hours relate to relatively small amounts of protein. In comparison, the IL-1ra production increases markedly between twenty-four and forty-eight hours in response to IgG, with levels rising from 5000pg/ml to 20000pg/ml in the 200μg/ml IgG group.

As a consequence of these studies, in D3 THP-1 cells, an incubation of at least forty-eight hours appears to be required for the maturation of an IL-1ra-inducing signal into significant protein secretion. In contrast, IL-1β levels appear to reach a peak more rapidly and then climb slowly over the duration of the time-courses studied. Consequently to compare the effect of a stimulus on the balance between IL-1ra and IL-1β production from D3 THP-1 cells an incubation of at least forty-eight hours was used.

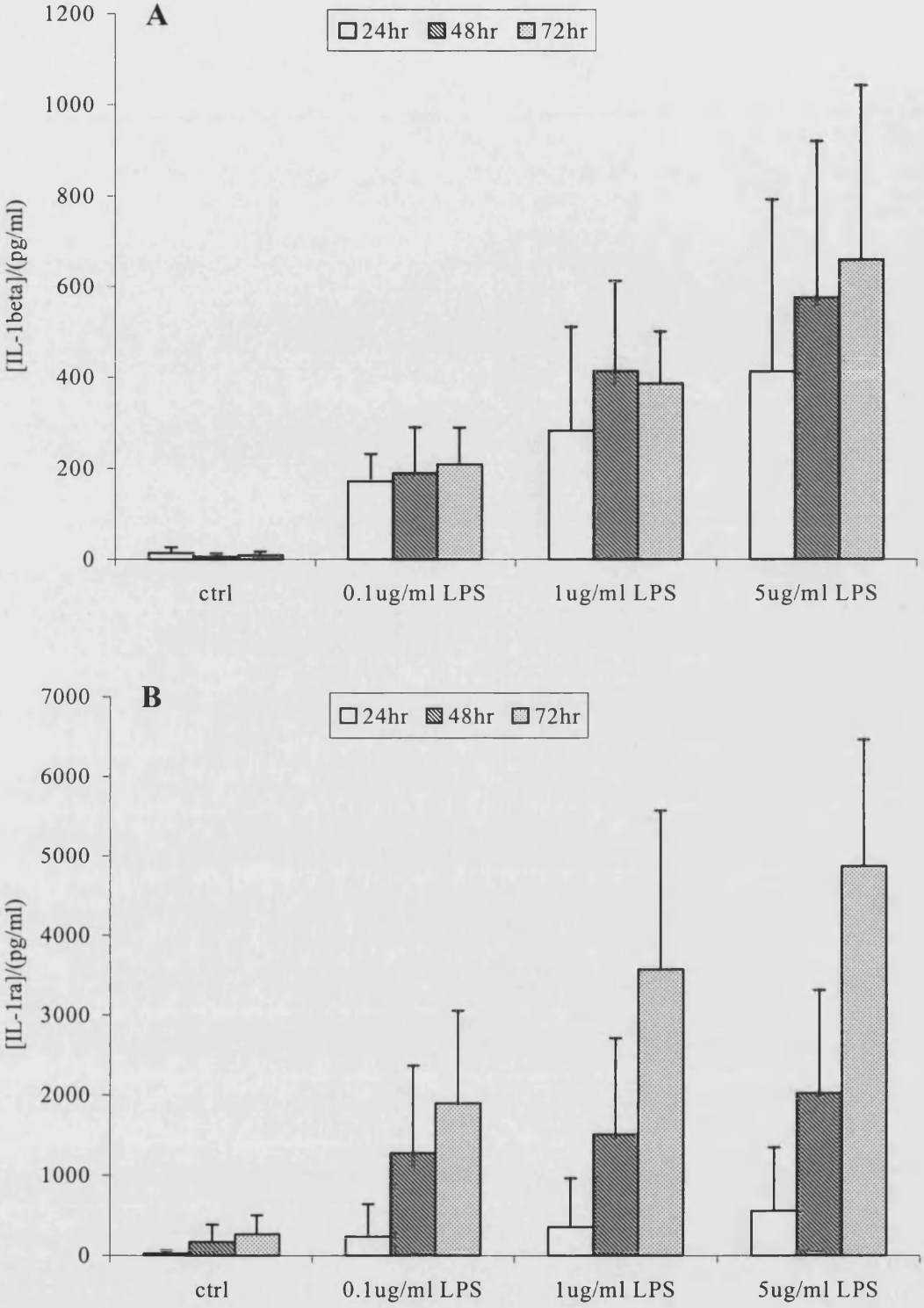


Figure 6.3: Effect of LPS on Cytokine Production by D3-Differentiated THP-1 cells.

Panel A: Shows IL-1 β release from 48 hour differentiated D3 THP-1 cells. Cells were cultured in the presence of LPS (0.1-5ug/ml) and cytokine secretion was determined over a three-day time-course.

Panel B: Shows the parallel IL-1ra content of the same supernatants (n=3±STDEV).

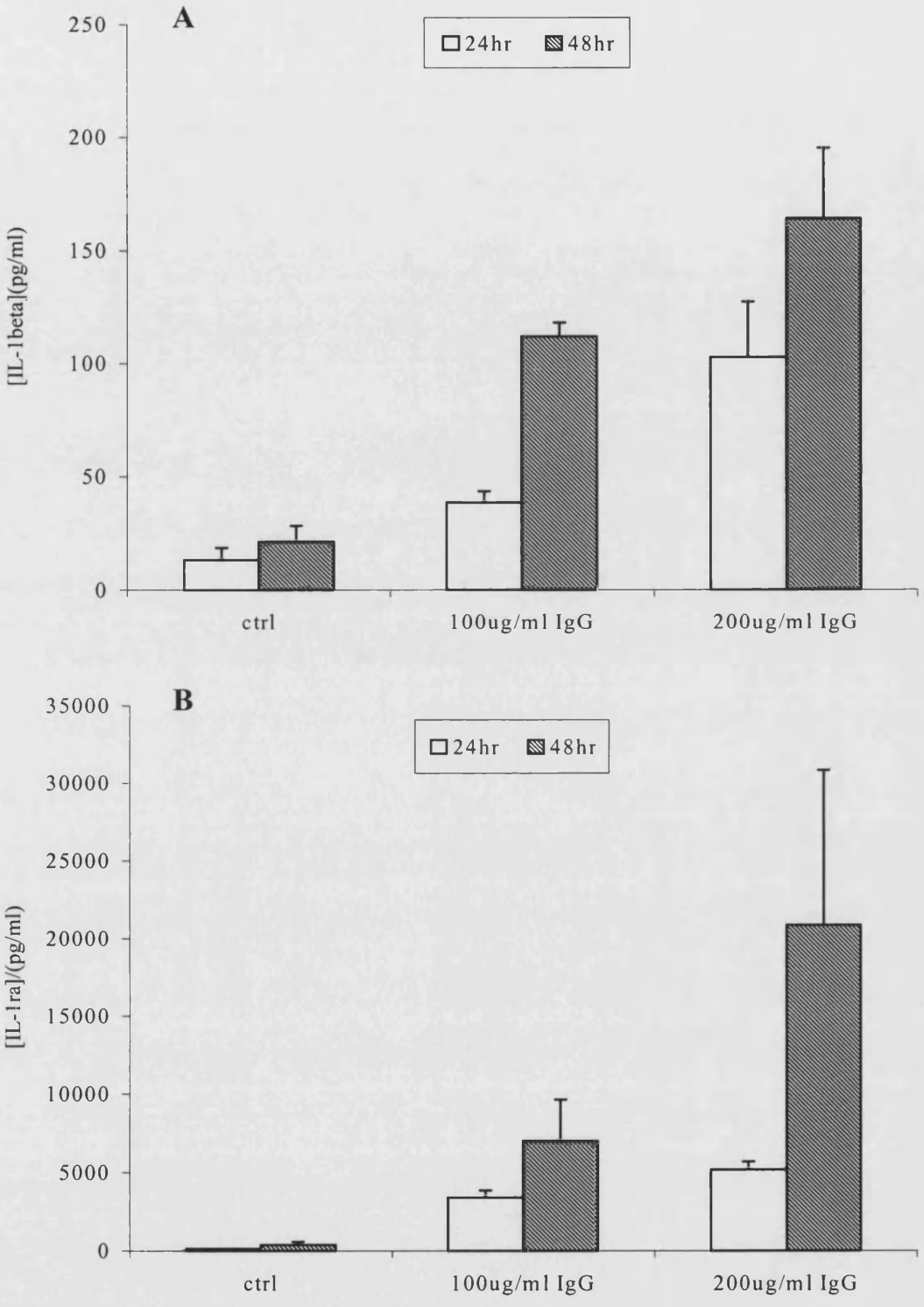


Figure 6.4: IgG Driven IL-1β and IL-1ra Release from D3 THP-1 Cells.
Panel A: Shows IL-1β release from 48 hour differentiated D3 THP-1 cells cultured on plastic-bound IgG. Supernatants were assessed for cytokine content after 24 and 48 hours of culture (n=3 ±STDEV).
Panel B: Shows parallel IL-1ra content of same supernatants.

6.2.3: Comparison of IL-1ra and IL-1β Production from PBMCs and Purified Monocytes

As well as using THP-1 cells as model monocytes for the interpretation of T cell derived signals and their role in regulating monocyte cytokine profiles a major goal of this study was to define monocytic responses to different T cell subsets. In order to be able to study the responses of mononuclear cells from different patient groups, protocols had to be defined to determine basic responses to positive control stimuli. Comparisons were made between simple PBMC preparations and purified resting monocyte preparations that had been harvested using MACS monocyte negative selection beads. As both IL-1β and IL-1ra are monocyte derived cytokines it was expected that purified monocytic cells would provide a cleaner experimental system to study. Consequently, the next experiments sought to look at basic LPS and IgG responses. PBMCs and monocytes were prepared as described in **method 2.2.4/5**, cultured at $1x10^6$/ml in RPMI-1640 and incubated in 200µl volumes in round bottom 96 well plates. Purified monocytes were routinely found to be greater than 90% CD14 positive whereas PBMCs varied between 10% and 25% CD14 positive.

Figure 6.5 shows a comparison between IL-1β (top panel) and IL-1ra (bottom panel) secretion from PBMCs and monocytes in response to a forty-eight hour incubation with LPS. In both the monocyte and PBMC preparations, in spite of adherence to the plastic of the culture plates, it can be seen that basal IL-1β release was low with levels of 20pg/ml and 57pg/ml respectively. IL-1β secretion was much more responsive to LPS than that seen in THP-1 cells with significant induction being observed with just 1ng/ml. Interestingly, the IL-1β response of PBMCs to LPS was not considerably different to that of purified monocytes despite the fact that the CD14 positive population was as much as nine fold smaller. In both cell populations maximal IL-1β induction was seen with 10ng/ml LPS with levels of 1751pg/ml in PBMCs and 2630pg/ml in monocytes. In contrast to IL-1β secretion, in these experiments LPS inhibited IL-1ra production. In control groups both cell populations produced high levels of IL-1ra, but compared to IL-1β secretion there was a large difference between the amounts of IL-1ra the two populations produced, with PBMCs secreting 6018pg/ml and monocytes secreting 13885pg/ml.

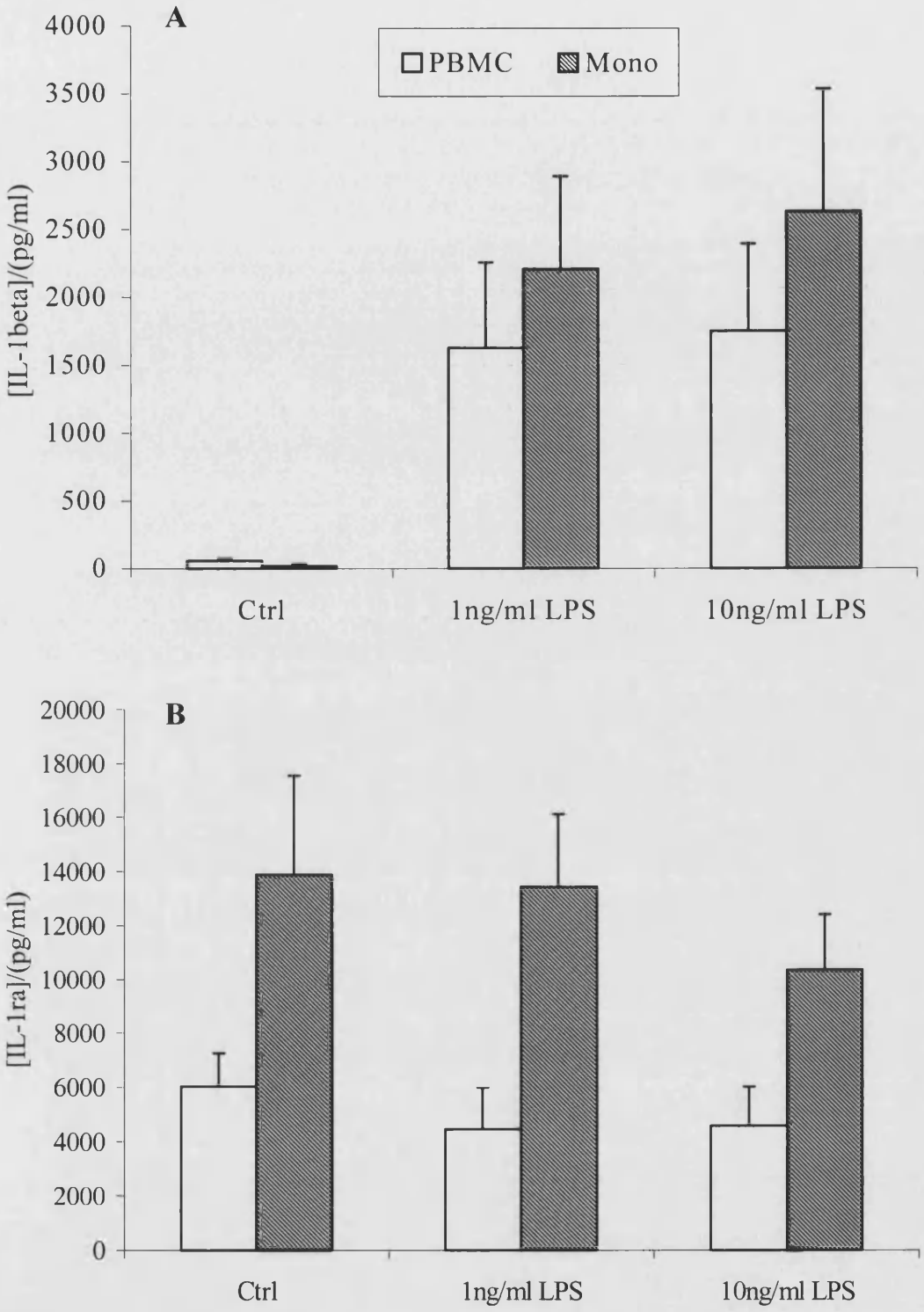


Figure 6.5: Comparison of Cytokine Production from Purified Monocytes and PBMCs in Responses to LPS.

Panel A: IL-1β production induced by LPS over a 48hr culture in 96 well plates (n=3±SEM).

Panel B: Parallel IL-1ra production induced by LPS over a 48hr culture in 96 well plates (n=3±SEM).

As can be seen in **figure 6.6**, similar to the results observed in D3 THP-1 cells, IgG had a much more dramatic effect on IL-1ra production than IL-1β production in both PBMCs and monocytes. In the purified monocyte population there was a concentration dependent increase in IL-1β production but the levels of protein secreted were very low with a maximum of 95pg/ml being achieved with 100μg/ml IgG stimulation. The PBMC population responded in a similar manner, but with slightly greater levels of IL-1β secretion. The IL-1ra responses to IgG stimulation were much more consistent than the IL-1β responses. In both the PBMCs and the monocytes IgG stimulation resulted in a concentration dependent increase in IL-1ra release. In PBMCs control levels of IL-1ra were measured at 6018pg/ml, rising to 15046pg/ml with 100μg/ml IgG stimulation. In monocytes, however, control levels of IL-1ra were measured at 13885pg/ml, rising to 24505pg/ml with 100μg/ml IgG stimulation.

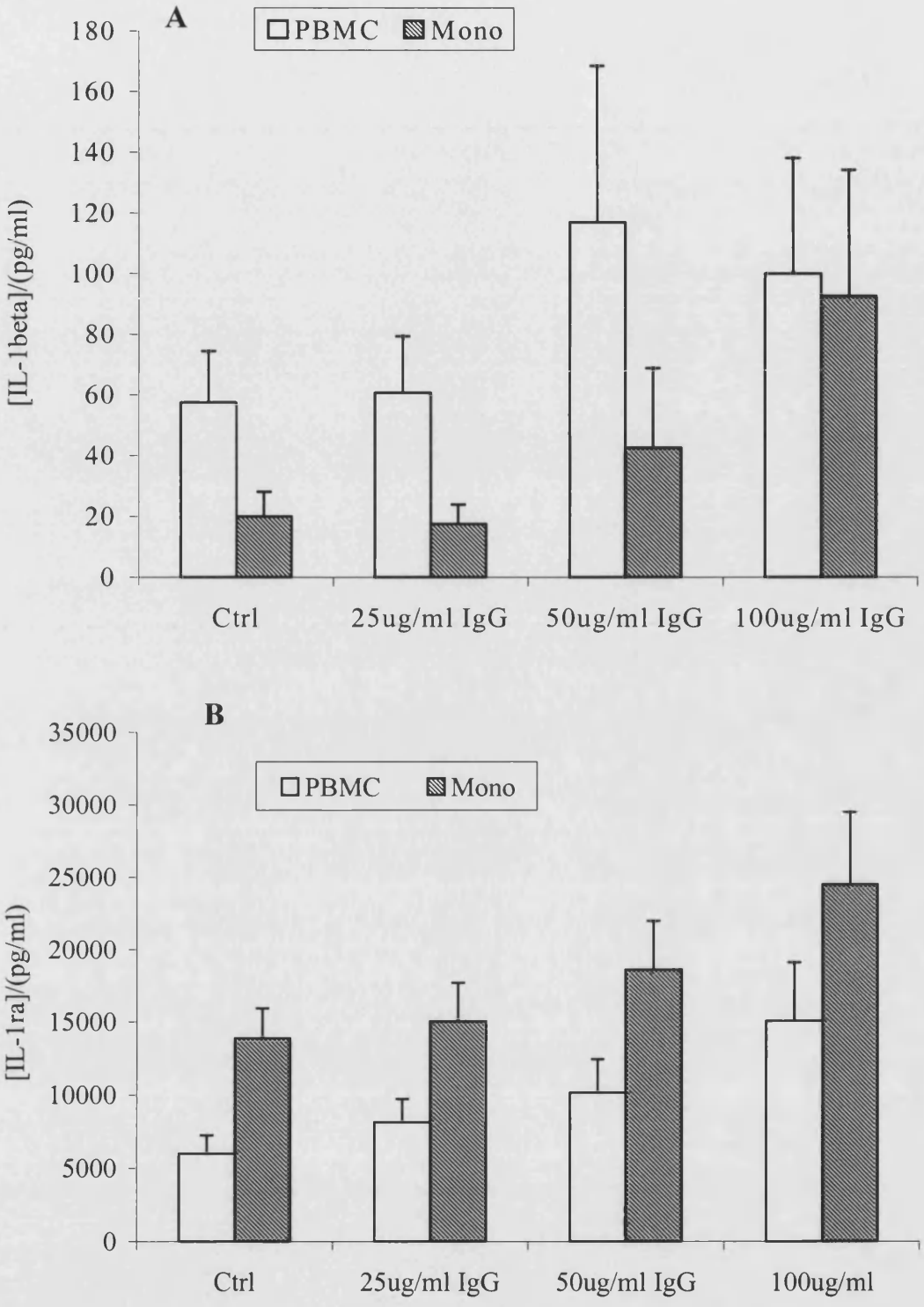


Figure 6.6: Comparison of Cytokine Production from Purified Monocytes and PBMCs in Responses to IgG.

Panel A: IL-1β production induced by plastic-bound IgG over a 48hr culture in 96 well plates (n=3±SEM).

Panel B: Parallel IL-1ra production induced by plastic-bound IgG over a 48hr culture in 96 well plates (n=3±SEM).

6.2.4: T cell Activation

Having tested and compared the suitability of THP-1 cells, monocytes and PBMCs for their IL-1β and IL-1ra responses to control stimuli the next step in the construction of a co-culture model was the definition of T cell activation. T cell activation is characterised by cell enlargement, proliferation and clonal expansion and the production of cytokines that mediate specific effector functions. For this to occur requires the presentation of an antigen and co-stimulation provided through the engagement of CD28 (432,450,451). CD28 is expressed on the surface of naive/resting T cells and interacts with molecules present on the APCs, that belong to the B7 receptor family (452,453). Extensive investigations have identified CD28 as an important T cell receptor in the process of activation. Studies using monoclonal antibodies for CD28 have clearly shown the co-stimulatory potential of this receptor. In this study, however, the natural ligand CD80 expressed in CHO cells was used. CHO cells were routinely assessed for CD80 expression using FACS analysis to assure co-stimulation (see **figure 6.7**). The ability of CD80 to synergise with TCR signals and induce T cell activation was examined. Signals of the TCR pathway were induced by aggregated CD3 antibodies, superantigen (SEA) and pharmacological agents like the phorbol ester PDBu and ionomycin. Phorbol esters activate Ras and PKC (454,455), which are downstream targets of TCR signalling and represent the calcium independent pathways of the TCR. Ras is a GTP binding protein and phorbol esters are thought to promote Ras activation by inhibiting GTPase activating proteins that restrict Ras activity (456,457). Ionomycin on the other hand increases calcium levels in the cell (454). T cell activation was quantified via the use of in *vitro* proliferation assays and analysis of surface expressed activation markers. Experiments were performed with freshly purified human resting T cells and the J16 human T cell line.

As can be seen in **figure 6.8**, unstimulated resting T cells proliferate at a very low rate. CD80 stimulation alone has a mild proliferative effect, but increasing the basal response to CD28 amplifies T cell responses triggered by pharmacological agents such as the phorbol ester PDBu and the calcium ionophore ionomycin (458). As **figure 6.8** shows PDBu alone at low concentrations was unable to induce proliferation of T cells. However, when combined with CD80 a strong proliferative response resulted, which was not observed with control CHO cells. At higher

concentrations, of 5ng/ml, PDBu alone induced mild proliferation but synergised strongly with CD80 co-stimulation.

As well as synergising with CD80, PDBu could also synergise with ionomycin, as shown in **figure 6.8B**. Although slight synergy was observed between 0.1ng/ml PDBu and CD80 in the previous experiment it appears that the combination of ionomycin with PDBu is even more potent at stimulating T cell proliferation. These results suggest that compared to CD80, the calcium induced pathways that ionomycin initiates can co-stimulate PDBu at lower concentrations. At higher concentrations of PDBu, however, the combination with ionomycin soon plateau's in its ability to synergise proliferative responses. With 0.5ng/ml PDBu and 1μM ionomycin the addition of CD80 co-stimulates the T cell response further. However, 5ng/ml PDBu with 1μM ionomycin has a comparable proliferative effect to 0.5ng/ml PDBu with 1μM ionomycin but now the addition of CD80 co-stimulation actually depresses T cell proliferation. From these data it appears that increased stimulation with phorbol ester and ionomycin may induce a phenotypic change in the T cell population in which CD80 has a negative regulatory function. Such negative regulation of T cell proliferation by CD80 may be a result of ligation to CTLA-4. CTLA-4 is a counter-regulatory relative of CD28 that delivers an opposing signal to the stimulated T cell (459).

As one of the aims of this study was to compare the effects of T cells from different patient groups for their ability to activate monocytes, these initial experiments defined activation responses of T cells from rheumatoid and normal samples. The proliferative responses of both rheumatoid and normal T cells were comparable when stimulated with phorbol ester ± ionomycin, with or without co-stimulation through CD28. When CD3 responses were studied, however, a markedly lower stimulation was observed in rheumatoid T cells. **Figure 6.9A** displays the considerably reduced response of sero-positive RA patients to CD3/CD80 ($P<0.05$-0.005) stimulation but comparable response to PMA/CD80 stimulation. This lack of CD3 responsiveness in these experiments was determined not to be due to decreased CD3 expression as surface FACS analysis confirmed equivalent expression between normal and SPRA patients (see **figure 6.10**).

The J16 cell line was also assessed for proliferative responses to phorbol ester, ionomycin, CD3 and CD80. Being a cell line, however, under resting conditions J16

cells proliferate at a considerable rate. Pharmacological stimulation of J16 cells with PMA and ionomycin actually leads to a decrease in proliferation. In contrast to PMA, however, direct stimulation of J16 through antibody aggregation of CD3 does not appear to modulate proliferation, even in combination with CD80 (see **figure 6.9B**).

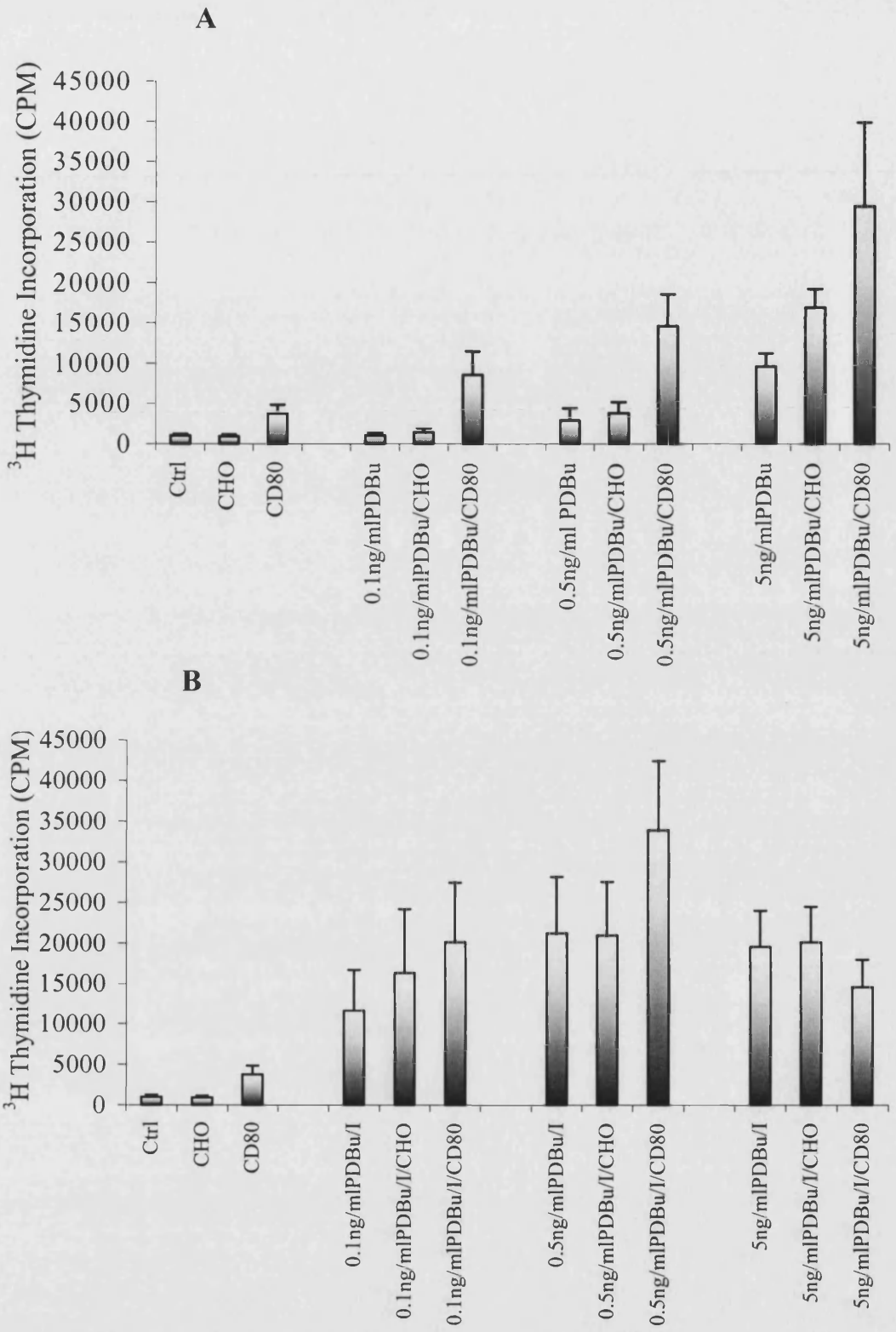


Figure 6.8: Activation Induced Proliferation of Resting T cells.
Panel A: Normal purified T cells were cultured for 72 hours with various concentrations of PDBu and co-stimulated with fixed CHO or CHO-CD80 cells. ^{3}H Thymidine incorporation was measured over the last 18 hours of culture (n=6-8±SEM).
Panel B: 1μM ionomycin was combined with the stimuli used in panel A (n=6-8±SEM).

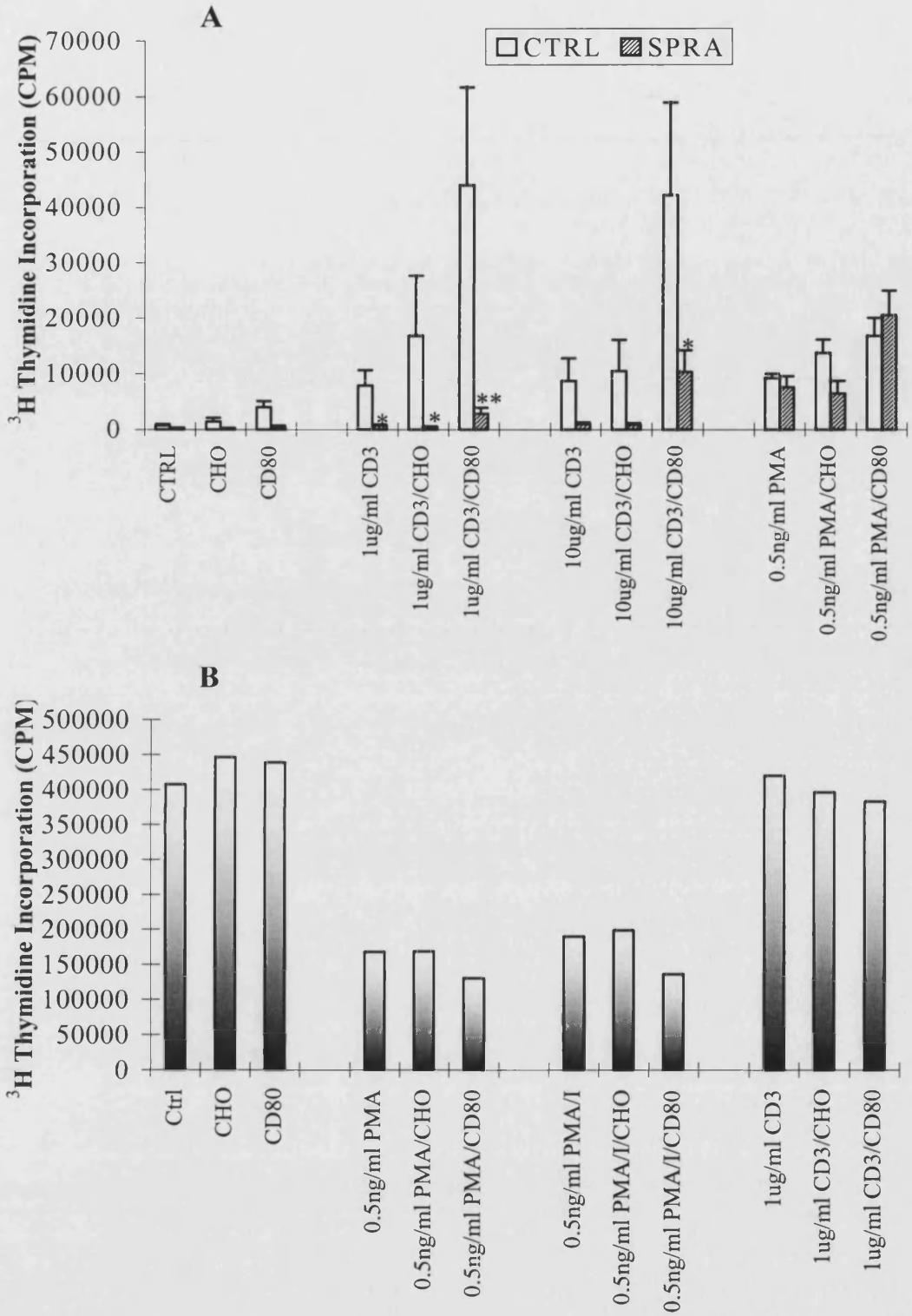


Figure 6.9 A: Reduced CD3/B7 Induced Proliferation In SPRA T cells n=5-9
T cells purified from RA patients and normal controls were stimulated for 72 hours with aggregated CD3 ± CD80. ^{3}H thymidine incorporation was measured over the last 18 hours of culture. PMA/CD80 stimulation was used as a positive control (*=P<0.05, **+P<0.005).
Panel B: Proliferative responses of Jurkat cell line to various stimuli. J16 cells were stimulated as described for normal T cells.

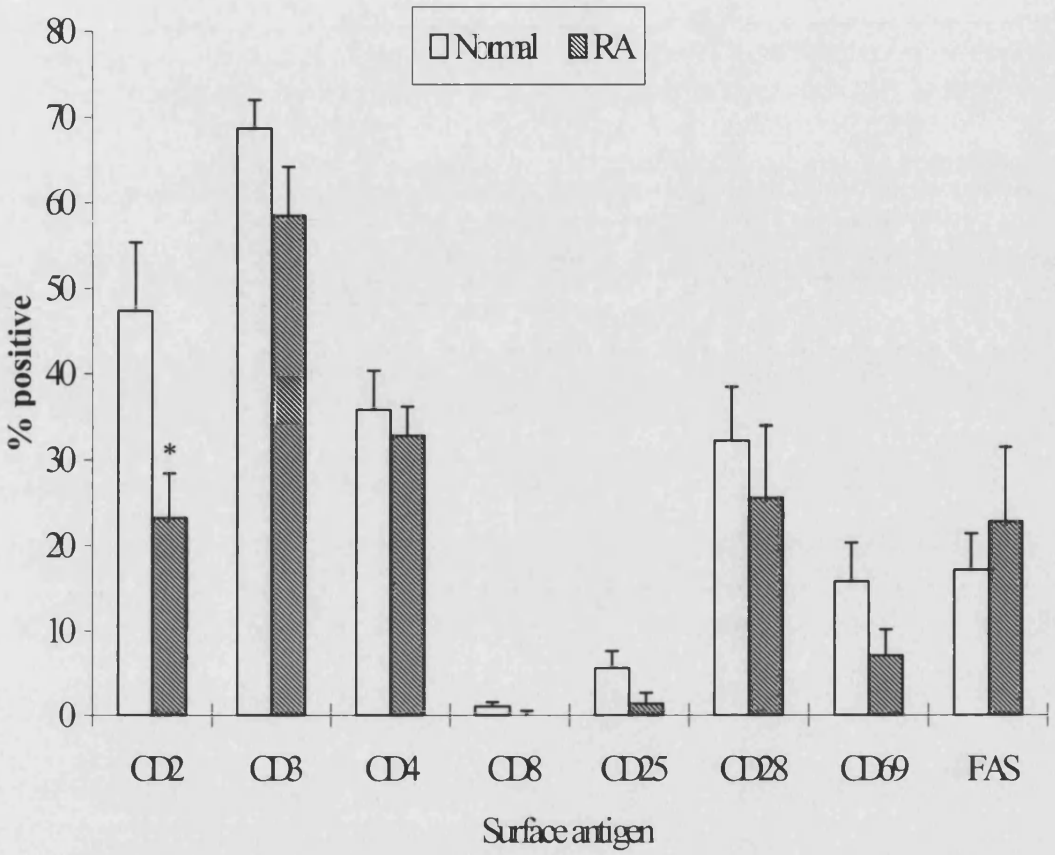


Figure 6.10: Surface antigen expression on normal and rheumatoid resting T cells.

Shows the percentage of the total population that stained positive for each particular surface antigen. This was determined by defining an area that would be considered positive, and measuring the number of cells that moved into this area upon staining (Norm n=9, RA n=7: *=P<0.05).

6.2.5: T Cell Surface Activation Markers

Interactions between T cell surface molecules and monocytes are critical for signal transduction in the immune response. As T cell membrane derived signals were being assessed for their ability to induce monocyte activation, T cell activation was also monitored in terms of surface activation markers.

Upon T cell activation several surface proteins are modulated including CD25, CD28, CD69 and CD95. The earliest activation marker to be upregulated is CD69 and as it has been implicated in the interaction between cell membranes in T cell-monocyte co-culture systems it was the molecule of most interest here. In this study CD69 expression was measured in order to quantify the degree of T cell, T cell blast and J16 activation induced by various stimuli.

Typical FACS plots for J16 and T cell activation marker expression are shown in **figure 6.11**. Although not all traces are shown in this figure it was observed that PDBu induced a concentration dependent increase in CD69 expression and that manipulating intracellular calcium levels with the ionophore ionomycin synergised to further enhance CD69 expression. Similarly, co-stimulation of the T cells with CHO-CD80 and PDBu also enhanced CD69 expression. In fact, the degree of surface CD69 expression induced correlated with the ability of the stimulus to induce T cell proliferation.

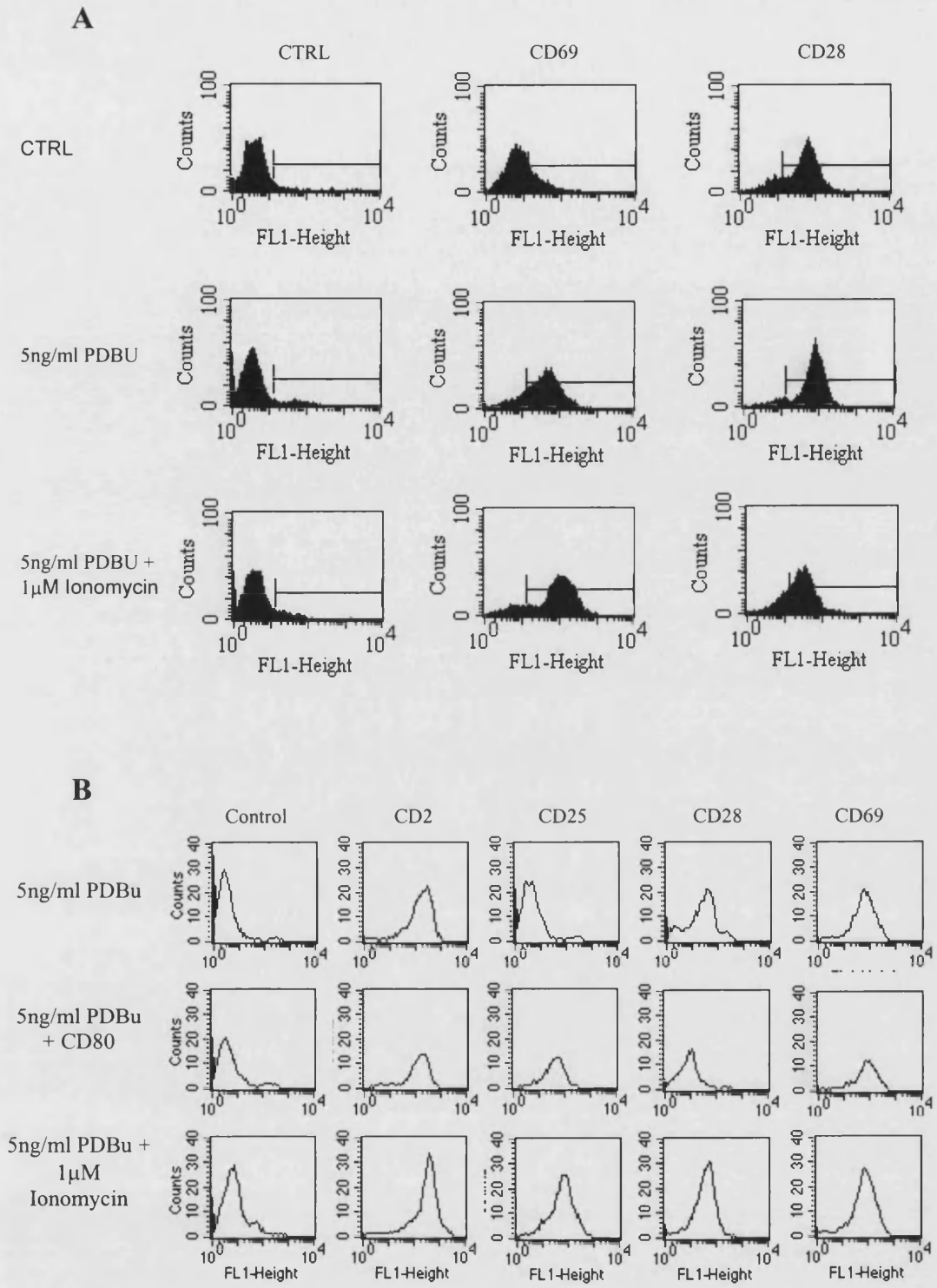


Figure 6.11: T cell Activation Surface Marker Expression.
Panel A: J16 cells were stimulated for 24 hours and assessed for surface marker expression using FACS analysis. Results are typical traces representative of three experiments.
Panel B: Purified T cells were stimulated for 24 hours and assessed for surface marker expression using FACS analysis. Results are typical traces representative of three experiments.

6.2.6: J16 Induced production of IL-1β and IL-1ra From Monocytes

Initial experiments to define basic monocyte responses to T cell stimulation used J16 cells as model T cells. Obviously, being a cell line, J16 cells vary greatly to resting T cells in terms of surface marker expression and proliferation. Upon activation with PDBu and ionomycin, however, J16 cells demonstrate a similar surface activation marker profile to normal T cells. Consequently, fixed activated and resting J16 cells were tested for their ability to deliver a membrane bound signal to monocytic cells. In the following experiments J16 cells were activated for eighteen hours with 5ng/ml PDBu and 1μM ionomycin, fixed with 0.025% glutaraldehyde and resuspended at a density of $5x10^6$/ml in RPMI-1640. Fixed J16 cells were cultured with monocytes at a ratio of 5:1, similar to that seen in peripheral blood, and incubated for forty-eight hours.

Figure 6.12 compares the responses of D3 THP-1 cells, purified monocytes and PBMCs, with IL-1β production being displayed in the top panel and IL-1ra production in the bottom panel. All three monocyte preparations responded to J16 co-culture. IL-1β secretion was induced in PBMCs and purified monocytes to a similar extent by both resting and activated J16 cells. In D3 THP-1 cells, however, resting J16 cells had no effect on IL-1β secretion but activated J16 cells did, with protein levels increasing from a control concentration of 11pg/ml to 105pg/ml. In all experiments, however, although J16 co-culture induced a significant increase in IL-1β release the actual levels were quite low compared to LPS stimulation. The IL-1ra response from all three cell preparations was quite different to the IL-1β response. D3 THP-1 cells produced IL-1ra in response to resting J16 cells but significantly more in response to activated J16 cells. The response from purified monocytes and PBMCs to J16 cells was not as dramatic as that of D3 THP-1 cells, perhaps due to adherence activation stimulating high control levels of IL-1ra. Monocytes particularly had a very high basal production of IL-1ra that was inhibited slightly by resting J16 cells and augmented slightly by activated J16 cells. In contrast, PBMCs gave a more consistent response with resting J16 cells having no effect on IL-1ra production but moderate induction occurring with activated J16 cells.

Control resting and activated J16 cultures themselves gave no IL-1β or IL-1ra production.

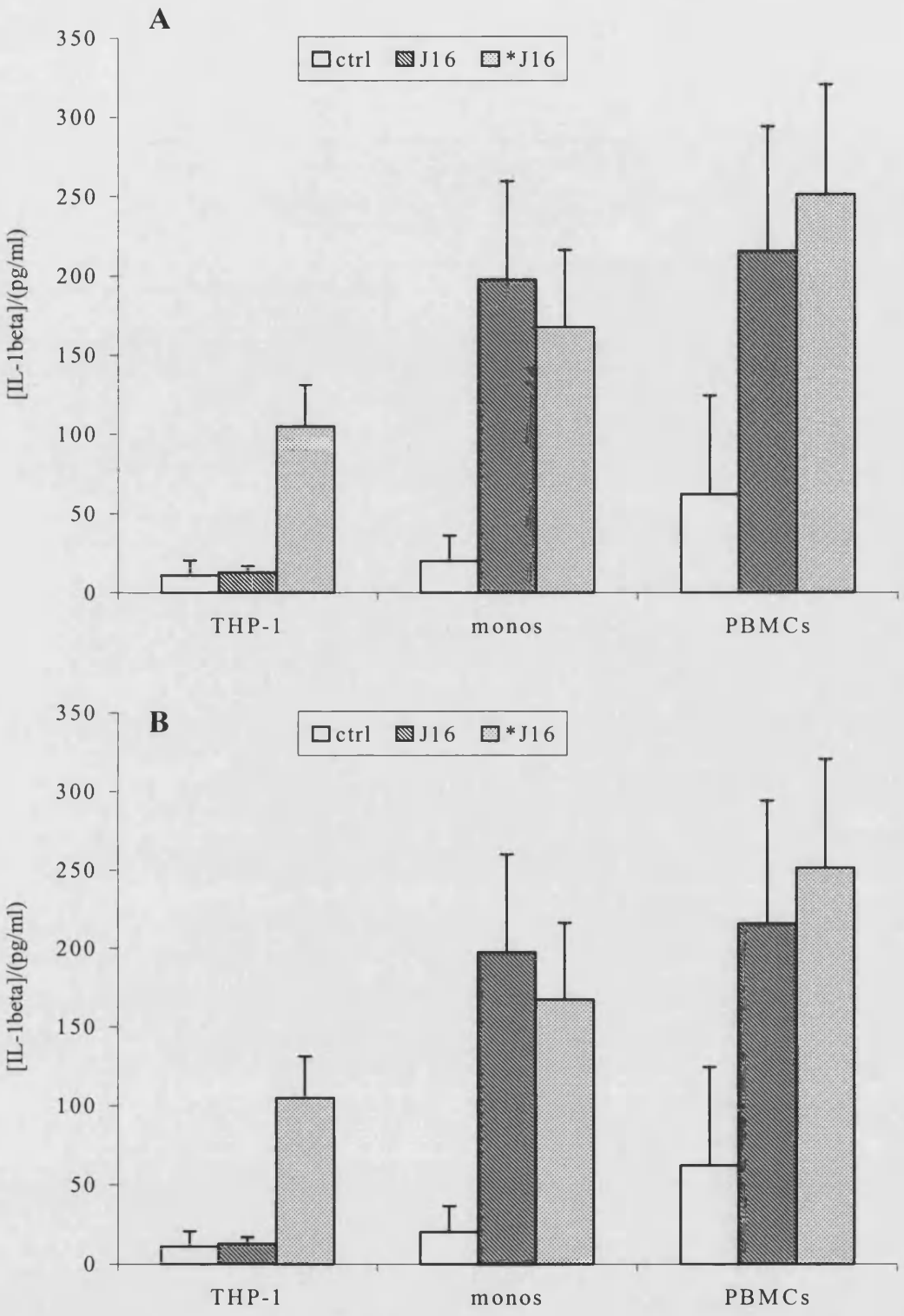


Figure 6.12: Comparison of Cytokine Production from Purified Monocytes , THP-1 and PBMCs in Responses to Stimulation with fixed J16 cells.
Panel A: 48 hour IL-1β release. Blood PBMCs, purified monocytes and the monocytic cell line D3 THP-1 were cultured with fixed resting J16s or fixed activated J16s (*J16). Cell supernatants were harvested after 48 hours (Monocyte n=3, THP-1 n=4, PBMCs n=6 ±SEM).
Panel B: Parallel IL-1ra release from same supernatants.

6.2.7: T cell Driven Monocyte IL-1β and IL-1ra Production

To test whether normal T cells would have the same effect as J16 cells on monocyte IL-1β and IL-1ra production an experiment was carried out in which half of a blood sample was purified to monocytes and the other half to T cells. The T cells were then activated with various stimuli before being fixed and then added back to the autologous monocytes. The cell co-culture was incubated for forty-eight hours and then the cytokine release into the supernatants determined. **Figure 6.13** displays the combined data of two experiments with IL-1β and IL-1ra release in the top and bottom panels respectively. Due to the fact that these data represents just two combined experiments it is difficult to read too much into it. However, what can be seen is that normal T cells have the ability to modulate the IL-1β and IL-1ra production of their autologous monocytes. In these figures, the control levels of cytokines produced are consistent with previous purified monocyte experiments with low IL-1β production and relatively high IL-1ra production. Co-culture of the monocytes with resting T cells has no effect on IL-1β production but does induce a large increase in IL-1ra release. T cells that have been activated with a proliferative stimuli, such as PMA/CD80 or CD3/CD80 gain the ability to induce IL-1β production but do not affect IL-1ra production particularly more than resting T cells. These data are of course preliminary so should not be over-interpreted. However interesting features persist, such as the fact that T cells that have been incubated with PMA, ionomycin and CD80, a stimulus linked with CTLA4 expression and down-regulation of proliferation, result in much less IL-1β induction than just PMA and ionomycin activated T cells.

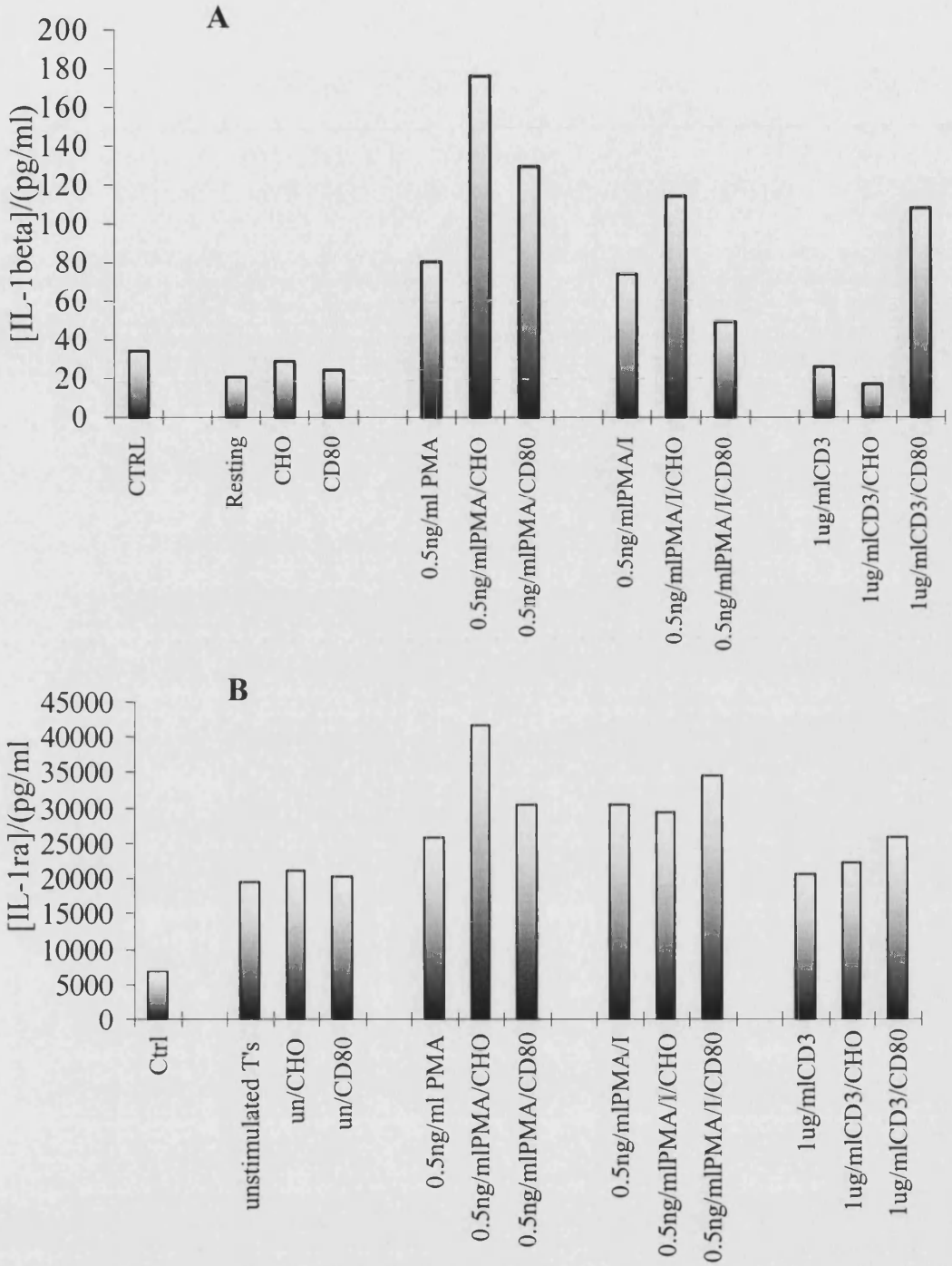


Figure 6.13: T cell Driven IL-1β and IL-1ra Production from Autologous Monocytes.

Panel A: 48 hour IL-1β release. Blood samples were split, half purified to monocytes and half to T cells. The T cells were stimulated with various combinations of PMA, ionomycin, CD3 and CD80 for 18 hours then fixed and re-added to the monocytes. Cell supernatants were harvested after 48 hours (n=2).

Panel B: Parallel IL-1ra release from same supernatants.

6.2.8: Effect of LPS and IgG on J16 Driven IL-1β and IL-1ra Production from D3 THP-1 cells

In resting D3 THP-1 cells the basal release of both IL-1ra and IL-1β is very low. Both of these cytokines have been shown to be highly inducible by IgG and LPS respectively and also modulated by T cell co-culture. Although J16 cells, particularly activated J16 cells, provide a strong inducing signal for the production of IL-1ra their effect on IL-1β release stimulates relatively small amounts of protein. The effect of J16 cells on basal IL-1β release from D3 THP-1 cells is significant but, compared to an LPS response, relatively minor. As a result, the aim of this section is to compare the effect of J16 co-culture on the responses of D3 THP-1 cells that have already been primed with IL-1β– or IL-1ra-inducing stimuli. In these experiments resting or eighteen-hour PDBu/ionomycin activated J16 cells were fixed and co-cultured with D3 THP-1 cells in the presence of various concentrations of LPS or in wells coated with IgG.

Figure 6.14 shows IL-1β and IL-1ra production, in the top and bottom panels respectively, from D3 THP-1 cells that have been co-cultured as described above. The control response to LPS shows a concentration dependent increase in IL-1β and IL-1ra release. In the top panel it can be seen that activated J16 cells alone produce a small but significant ($P<0.05$) increase in IL-1β production from D3 THP-1 cells but that the magnitude of this is almost lost in the synergy that occurs when LPS is added to the culture. The maximum response in control D3 THP-1 cells to 5μg/ml LPS is 604pg/ml. Activated J16 cells alone induce 105pg/ml IL-1β but the combination of activated J16 and 5μg/ml LPS results in an induction of 4122pg/ml IL-1β. The synergy between activated J16 and LPS is dependent upon the concentration of LPS but even at 0.1μg/ml LPS an induction of 2276pg/ml is seen compared with a control secretion of 189pg/ml. This synergistic stimulation of IL-1β production was not mirrored by IL-1ra release in this study. Although IL-1ra is potently induced by both LPS and J16 cells (especially activated J16 cells) when cultured alone, addition of LPS to J16 stimulated monocytes has no effect on IL-1ra levels.

Interestingly, IgG addition to the D3 THP-1/J16 co-culture also affected the IL-1β and IL-1ra levels produced, as is shown in **figure 6.15**. The effects of IgG, however, were quite different with mild synergy being seen with J16 induced IL-1β and an

additive effect being seen with J16 induced IL-1ra. Where LPS had no effect on activated J16 induced IL-1ra, with levels remaining at about 6500pg/ml, 200μg/ml IgG raised the response to 14245pg/ml. It must be stressed, however, that although the synergy between IgG and J16 driven IL-1β is clear, again it concerns quite low levels of protein secretion especially compared to the combination of LPS and activated J16.

6.2.9: Effect of Fixation on J16 Induced Production of IL-1β and IL-1ra from D3 THP-1 cells and normal PBMCs

In all the experiments detailed so far, using co-culture to stimulate monocyte production of IL-1β and IL-1ra, the T cells have been fixed in order that only membrane interactions could occur. The following experiment sought to compare the effect of fixation on the ability of resting and activated J16 cells to induce D3 THP-1 production of IL-1β and IL-1ra and synergise with LPS stimulation. **Figure 6.16** shows IL-1β release and IL-1ra release, after a forty-eight hour incubation, in the top and bottom panels respectively. Unfortunately the data presented in this figure confuses the message that was derived from **figure 6.14**. In contrast to the previous experiment, which only saw synergy between LPS and fixed J16 cells in inducing IL-1β production, this experiment also shows synergy in the stimulation of IL-1ra release. This response is unexplainable and could be due to many factors including the state of differentiation of the THP-1 cells, the culture conditions of the J16 cells or the condition of the LPS used. Despite all attempts to keep conditions absolutely constant something had obviously changed in this system and affected the results accordingly. The important thing here, however, is that despite this inconsistency the data presented in **figure 6.16** still displays an interesting difference between the responses induced by fixed compared to unfixed J16 cells. It appears that unfixed J16 cells, especially activated J16 cells deliver a more potent IL-1β inducing signal and synergise more greatly with LPS when compared to fixed J16 cells. In contrast, fixation of the J16 cells leads to a far greater ability to induce IL-1ra production ($P<0.05$). It appears from these data that membrane-derived interactions and soluble mediators from T cells may differentially regulate production of IL-1β and IL-1ra from differentiated THP-1 cells. Interestingly, unfixed resting J16 cells significantly

inhibited LPS induced IL-1ra release ($P<0.05$) even though they had little effect upon LPS induced IL-1β.

Figure 6.17 shows the effect of fixation on J16 cell stimulation of PBMCs. Unfortunately as this figure only represents two experiments significant conclusions can't be made. The data, however, shows complementary trends to the THP-1 cell responses and is therefore worthy of discussion. Panel A shows the IL-1β release from PBMCs cultured with fixed and unfixed resting and activated J16 cells. Similarly to the D3 THP-1 cell responses a marked synergy is seen between the unfixed resting of activated J16 cells and LPS in stimulating IL-1β release from PBMCs. Fixation of the J16 cells practically negates this synergy, with IL-1β production being comparable to LPS alone. Due to high basal release of IL-1ra from the PBMCs it is difficult to interpret the effect of fixation on J16 stimulated PBMC IL-1ra production.

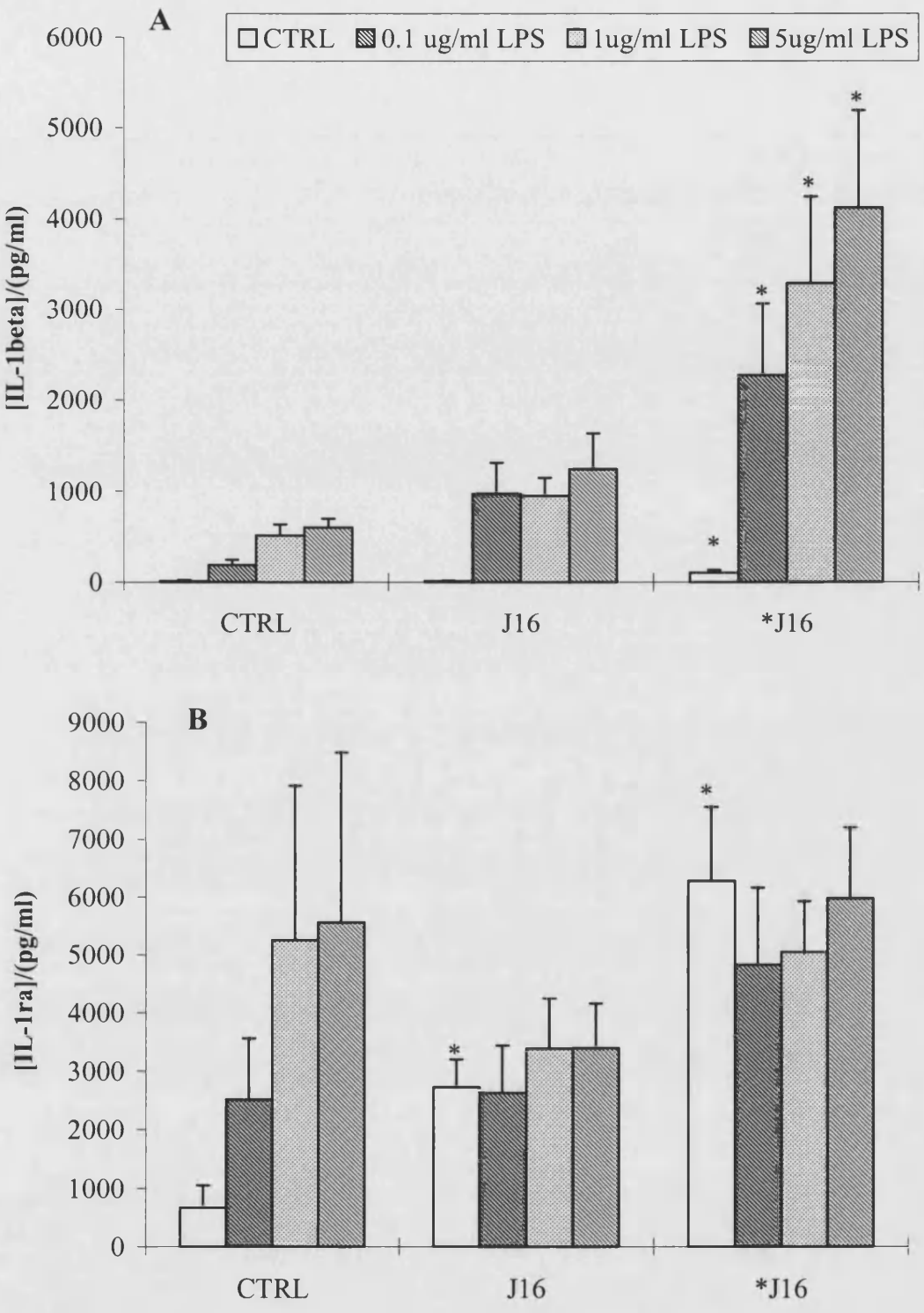


Figure 6.14: Effect of LPS on J16 Stimulated Cytokine Release from D3 THP-1 Cells.

Panel A: 48 hour IL-1β release. D3 THP-1 were cultured with fixed resting J16s or fixed activated J16s (*J16) with different concentrations of LPS. Cell supernatants were harvested after 48 hours (n=4-8±SEM: *=P<0.05).

Panel B: Parallel IL-1ra release from same supernatants.

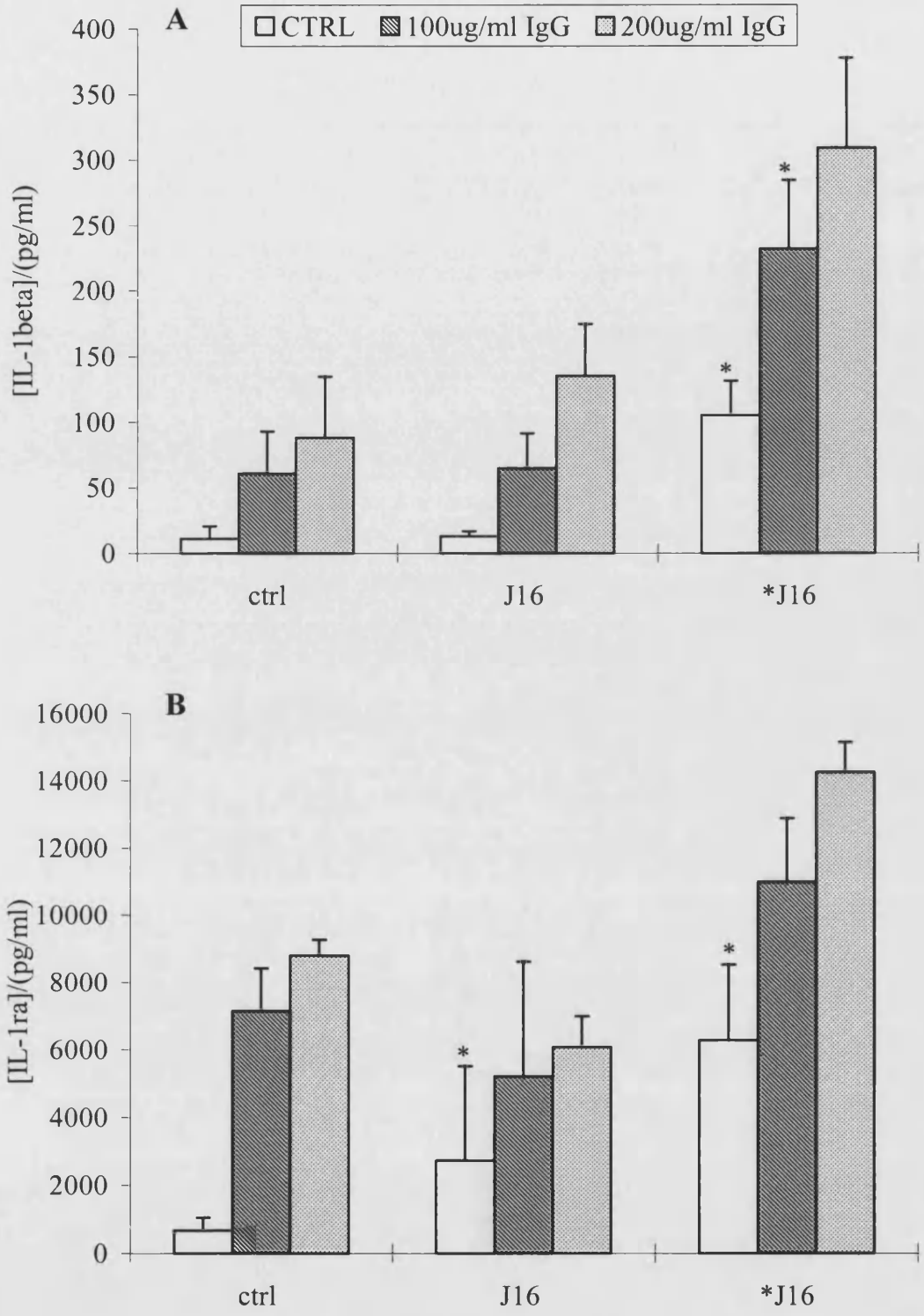


Figure 6.15: Effect of IgG on J16 Stimulated Cytokine Release from D3 THP-1 Cells.

Panel A: 48 hour IL-1β release. D3 THP-1 were cultured with fixed resting J16s or fixed activated J16s (*J16) on plates coated with different concentrations of IgG. Cell supernatants were harvested after 48 hours (n=3-4 ±SEM: *=P<0.05).

Panel B: Parallel IL-1ra release from same supernatants.

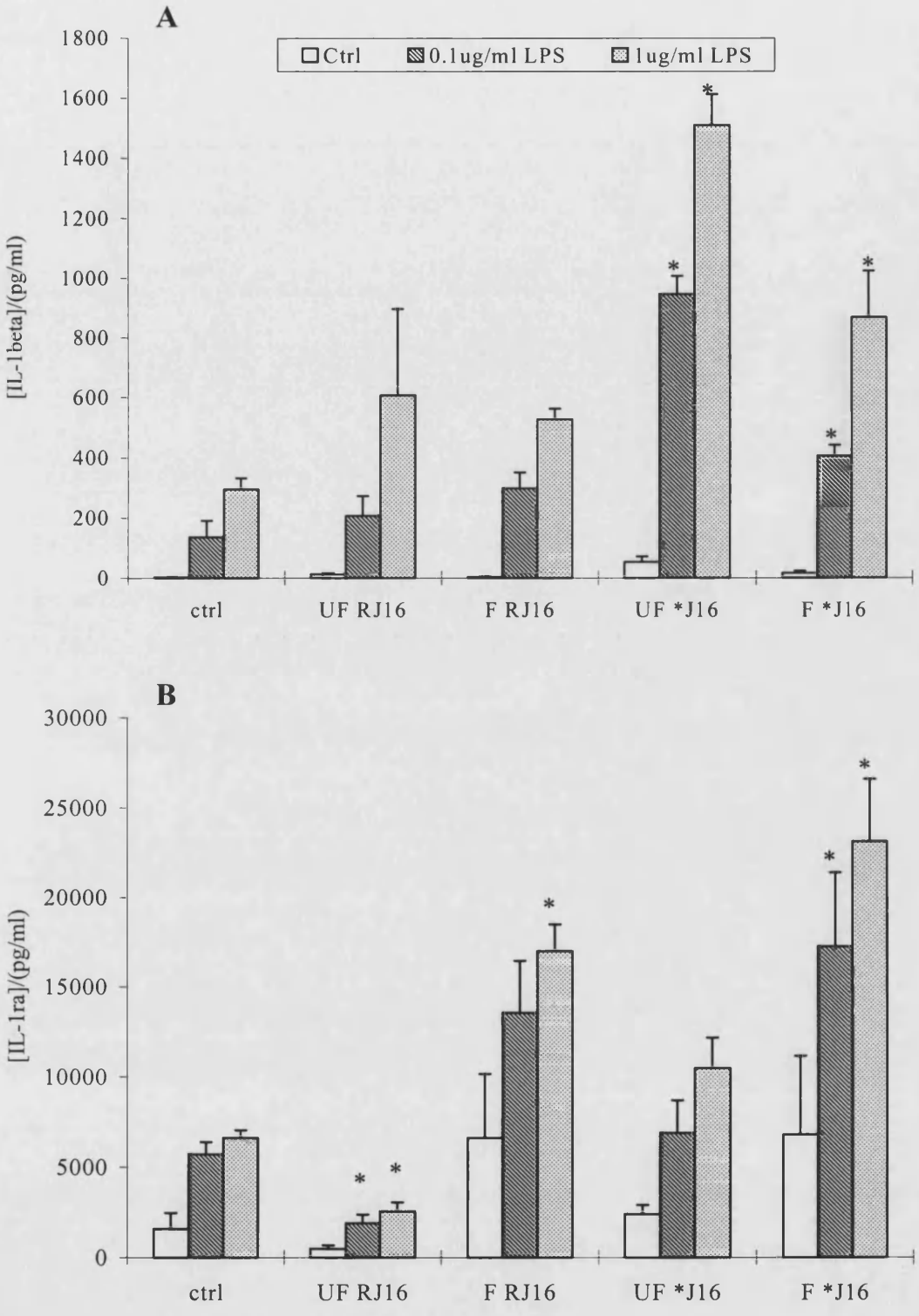


Figure 6.16: Effect of Fixation on J16 Stimulated Cytokine Release from D3 THP-1 Cells.

Panel A: 48 hour IL-1β release. D3 THP-1 were cultured with fixed/unfixed resting J16s (F/UFRJ16) or fixed/unfixed activated J16s (F/UF*J16) with different concentrations of LPS. Cell supernatants were harvested after 48 hours (n=4-7 ±SEM: *=P<0.05).

Panel B: Parallel IL-1ra release from same supernatants.

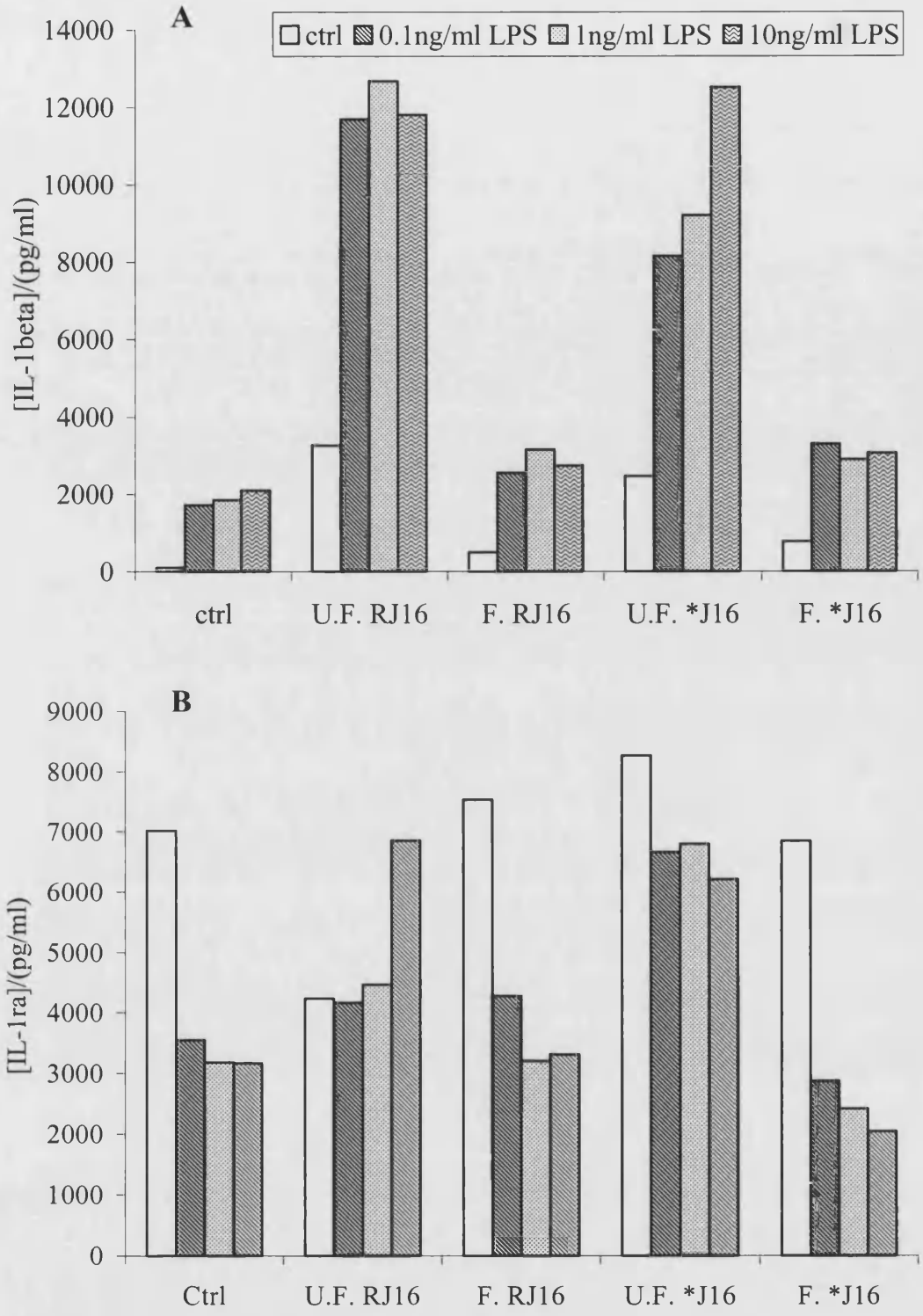


Figure 6.17: Effect of Fixation on J16 Stimulated Cytokine Release from PBMCs.

Panel A: 48 hour IL-1β release. Normal PBMCs were cultured with fixed/unfixed resting J16s (F./U.F.RJ16) or fixed/unfixed activated J16s (F./U.F.*J16) with different concentrations of LPS. Cell supernatants were harvested after 48 hours (n=2).

Panel B: Parallel IL-1ra release from same supernatants.

6.2.10: Effect of T cell Blasts on Production of IL-1β and IL-1ra from D3 THP-1 cells

In light of the apparent synergism between LPS and T cell derived signals seen in the J16/D3 THP-1 co-culture system attempts were made to complement these data using normal T cells. A problem occurred, however, in that the T cell purification system used occasionally left a small monocyte contamination. Consequently the large concentrations of LPS required for IL-1β responses in D3 THP-1 cells induced a considerable level of IL-1β release from the contaminating monocytes. As a result, instead of studying the effects of resting T cells on cytokine production from D3 THP-1 cells activated T cell blasts were used. Unfortunately, due to time constraints the data presented in following figures is based on just one experiment. Consequently no clear conclusions can be drawn from these experiments but interesting observations are worthy of discussion.

The first experiment used T cell blasts generated by PDBu/ionomycin stimulated PBMCs, PDBu/ionomycin stimulated T cells and SEA stimulated PBMCs, co-cultured with D3 THP-1 cells and compared surface FACS data to cytokine induction profiles on days three, five and seven. Co-cultures were incubated for forty-eight hours with various concentrations of LPS. From day three onwards all of the T cell blast populations used to stimulate the THP-1 cells were found to be CD14 negative and when incubated with LPS did not produce either IL-1β or IL-1ra themselves.

Over the three time-points of T cell activation studied it was found that there was quite a variation in the amount of IL-1β and IL-1ra production induced in the D3 THP-1 cells. Looking at the surface marker expression in the T blasts (bottom panels of each page, **figures 6.18 to 6.20**) CD25 is up-regulated in all groups on day three and steadily drops over the time-course of the experiment. Similarly, CD69 is elevated on day three in all groups and also declines on day five and day seven after activation. In contrast, CD28 and CD95 levels remain fairly constant for the duration of the time-course. It is difficult to say how the surface marker expression relates to the induction of monocyte cytokines in this study, especially as the data is based upon one experiment. When analysing the cytokine production, however, there are some trends that are interesting if not conclusive. On each of the three time-points all three T cell populations displayed an ability to induce both IL-1β and IL-1ra

production from D3 THP-1 cells. On day three, all three blast groups induced equivalent amounts of IL-1β and all displayed synergy with LPS in the induction of both IL-1β and IL-1ra. The synergistic response to 1μg/ml LPS and T cell blast co-culture yielded an IL-1β production of 600 to 700pg/ml. In comparison, the IL-1ra responses seemed much greater, with up to 7000pg/ml being produced in the PIP + LPS group. The IL-1ra production measured in response to stimulation with day three blasts was higher than that using day five or seven. The degree of IL-1ra production correlated to some extent with the surface expression of CD69 on the blasts. The control IL-1β response to LPS in this particular experiment was a little low, however, with only 216pg/ml being produced in response to 1μg/ml LPS. Usual D3 THP-1 IL-1β responses to 1μg/ml LPS range between 300 and 500pg/ml. In contrast, the IL-1ra response to LPS was a little higher than usual with about 1000pg/ml being produced compared to a more usual value of between 600 and 800pg/ml. The balance between IL-1β and IL-1ra in the controls of this experiment highlight the difficulty in interpreting these data as in the day five experiment control responses are a little different. As can be seen in **figure 6.19**, the control response to LPS is a little higher in terms of IL-1β production and a little lower in terms of IL-1ra production. The differences are not great however but the trend in the blast co-cultures is the same, with increased IL-1β and reduced IL-1ra. The magnitude of the responses to T cells in **figure 6.19**, however, indicates that the variations in the control levels of cytokines produced are not sufficient to negate the blast induction data. The elevation in the control IL-1β response to LPS is not as large as the increased response to T cells, especially the PIP cells. The PI activated PBMC generated T cell blasts are particularly good at inducing IL-1β and IL-1ra and interestingly also appear to be the most activated T cell population, with highest levels of CD69 expression. There is also quite a clear correlation between the CD69 expression in the other T cell blasts and their ability to induce IL-1ra production. The day seven blast co-culture data also displays a little variation in control LPS responses, with average IL-1β production but quite high IL-1ra production. In this experiment, however, as can be seen in **figure 6.20**, there is a very large IL-1β response to SEA blasts, which is paralleled with a lesser IL-1ra response. In general, the high control LPS induced IL-1ra production was not affected by the addition of any of the T cell blasts and all blasts, except those generated with SEA, were poor

inducers of IL-1β. The profile of IL-1ra induction again correlated with CD69 expression between the blast groups and in general the low induction of IL-1ra reflected low levels of T cell activation in the day seven blasts.

The large induction of IL-1β and parallel reduction in IL-1ra production seen using T cells generated from SEA blasted PBMCs to stimulate D3 THP-1 cells prompted further investigation. Again, only one experiment is displayed here but the dramatic effects observed warrant mention. The goal of the experiment was to compare the data using fixed and unfixed J16 cells to stimulate D3 THP-1 cells with that using T cell blasts. The results with the J16 cells suggested the possibility that unfixed J16 cells modulate monocyte responses in a different manner to fixed J16 cells. Upon activation, fixed J16 cells adopt the ability to induce both IL-1β and IL-1ra production from D3 THP-1 cells. During the co-culture stimulation fixation was used to rule out the contribution of soluble mediators and limit responses to membrane interactions only. As can be seen from **figure 6.16**, membrane signals from J16 cells, in the absence of soluble mediators, favour the production of IL-1ra. When activated unfixed J16 cells are co-cultured with D3 THP-1 cells in combination with LPS there is a large induction of IL-1β production that is lost upon fixation. In this experiment, the ability of live T cell blasts to synergise with LPS and give a more significant induction of IL-1β production was tested.

PBMCs were prepared and activated with PHA, P/I or SEA. After six days the T cells that had been generated were taken and split into two groups. Half of the cells were fixed with 0.025% glutaraldehyde and the other half left unfixed. Both were resuspended in fresh RPMI-1640 at $5x10^6$/ml. Co-cultures were set-up as described previously using either fixed or unfixed blasts with various concentrations of LPS. All blasts were determined CD14 negative and control cultures of all blasts, in the absence of THP-1 cells gave no IL-1β or IL-1ra response to LPS. FACS analysis of the surface antigen expression of the different blast populations is shown in **figure 6.21**.

Unfortunately, due to the magnitude of the IL-1β response to unfixed SEA blasts in combination with LPS the rest of the IL-1β results displayed in **figure 6.22** are hard to discern. However, as the SEA response is the main observation of this experiment there is no need to extend the graphic. As far as IL-1β is concerned the D3 THP-1

cells responded well to LPS alone and the addition of all of the blasts resulted in an induction of protein production. The unfixed blasts, however, although not dramatically better at inducing IL-1β production alone synergised most greatly with LPS stimulation. Of the three blasting protocols studied the unfixed SEA blasts gave a five-fold greater synergy, with LPS for the induction of IL-1β, than any other of the T cells. Although only representing data from one experiment there are clear indications that soluble factors derived from T cells synergise with LPS stimulation in inducing IL-1β production.

All of the unfixed T cell blasts were also able to induce IL-1ra production but similarly to IL-1β production, the clearest IL-1ra induction was seen when the THP-1 cells were stimulated with unfixed T cells. These results are opposite to what is seen with J16 cells where fixation increases the monocyte IL-1ra response. The three different blast populations were equivalent in their ability to induce IL-1ra production but the SEA blast synergy with LPS was not as marked as that seen with PHA and P/I blasts. From these data it appears that SEA blasts, when unfixed and co-cultured with D3 THP-1 cells synergise with LPS stimulation to favour induction of IL-1β.

CD69 expression correlates with the abitity of T cells to induce monocyte IL-1ra production ($R=0.727$, $p=0.024$) but doesn't correlate with IL-1beta induction ($R=-0.383$, $p=0.323$).

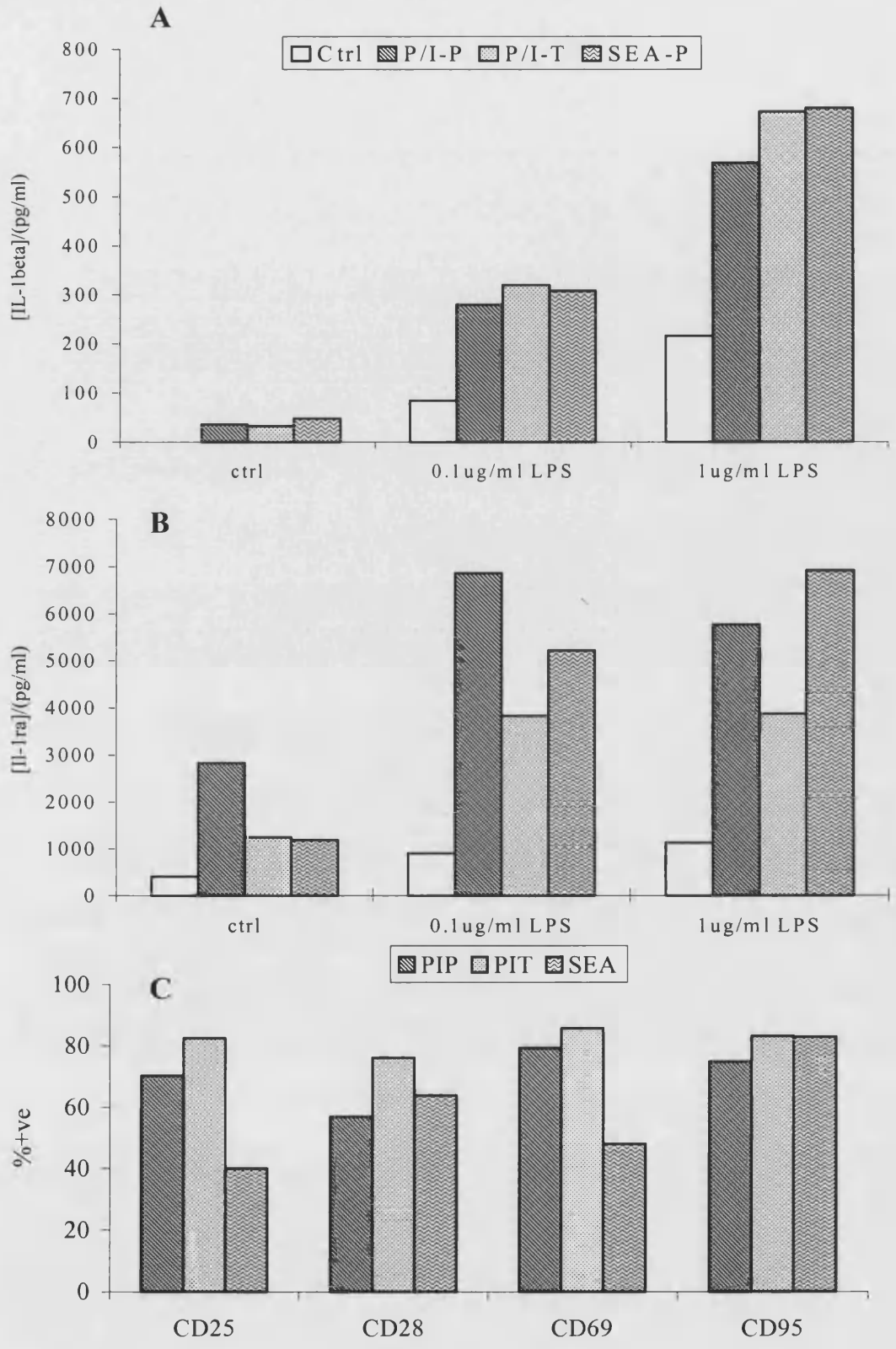


Figure 6.18: Day 3 blasts co-cultured with D3 THP-1 Cells for 48 Hours.
Panel A: IL-1β release from D3 THP-1 cells stimulated with fixed T cell blasts and various concentrations of LPS (n=1).
Panel B: Parallel IL-1ra release from same supernatants.
Panel C: FACS analysis of surface antigen expression.

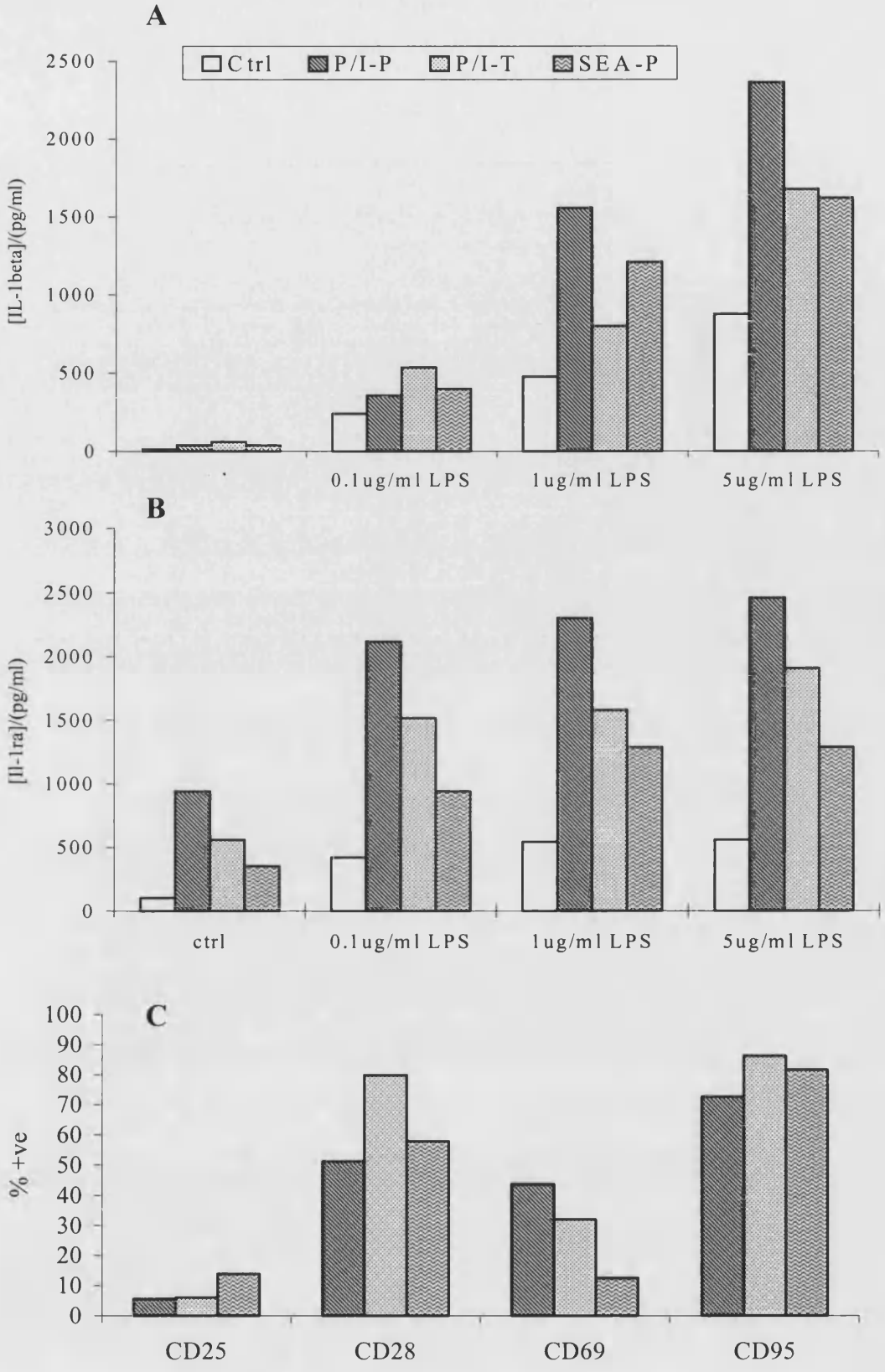


Figure 6.19: Day 5 blasts co-cultured with D3 THP-1 Cells for 48 Hours.
Panel A: IL-1β release from D3 THP-1 cells stimulated with fixed T cell blasts and various concentrations of LPS (n=1).
Panel B: Parallel IL-1ra release from same supernatants.
Panel C: FACS analysis of surface antigen expression.

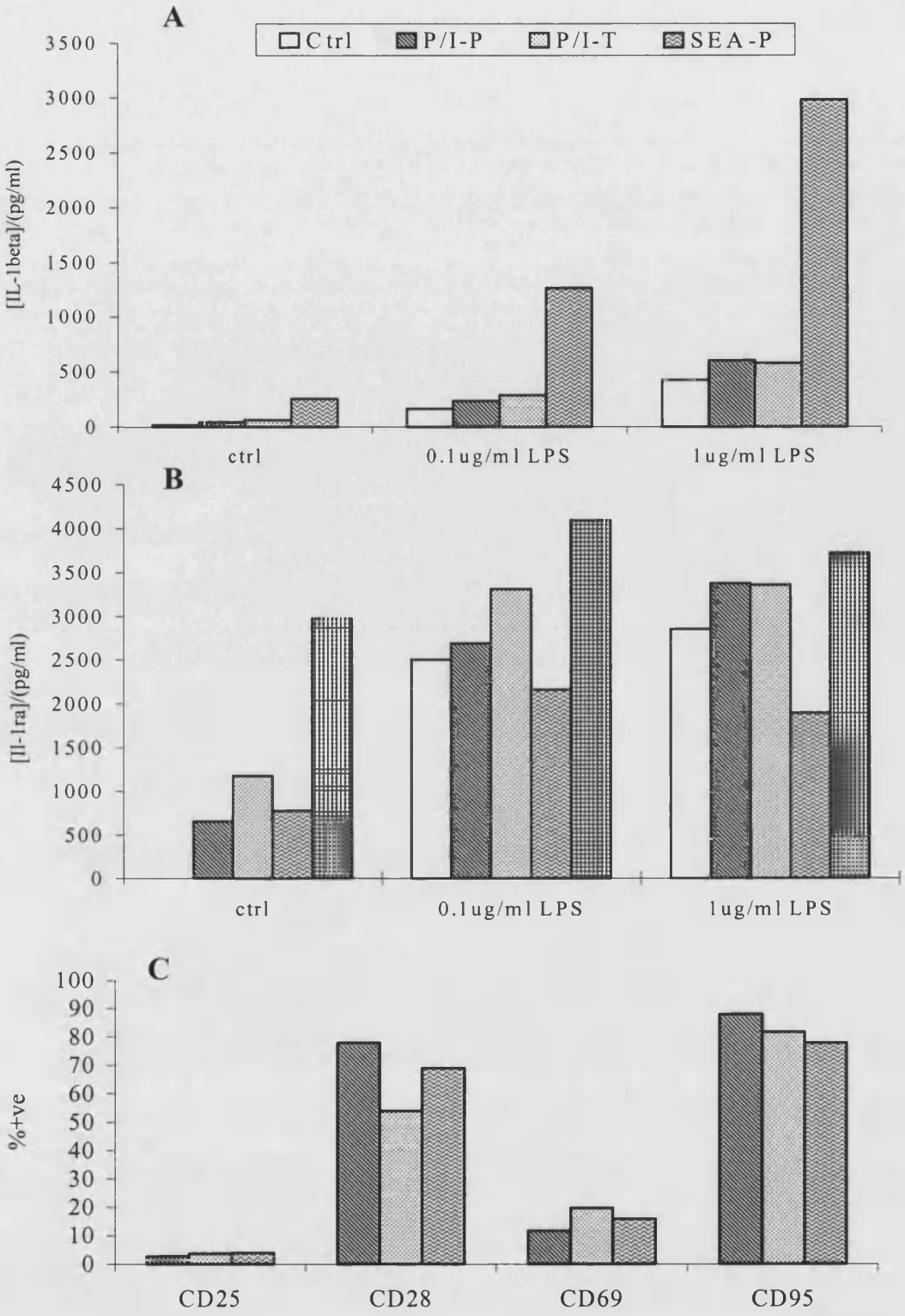


Figure 6.20: Day 7 blasts co-cultured with D3 THP-1 Cells for 48 Hours.
Panel A: IL-1β release from D3 THP-1 cells stimulated with fixed T cell blasts and various concentrations of LPS (n=1).
Panel B: Parallel IL-1ra release from same supernatants.
Panel C: FACS analysis of surface antigen expression.

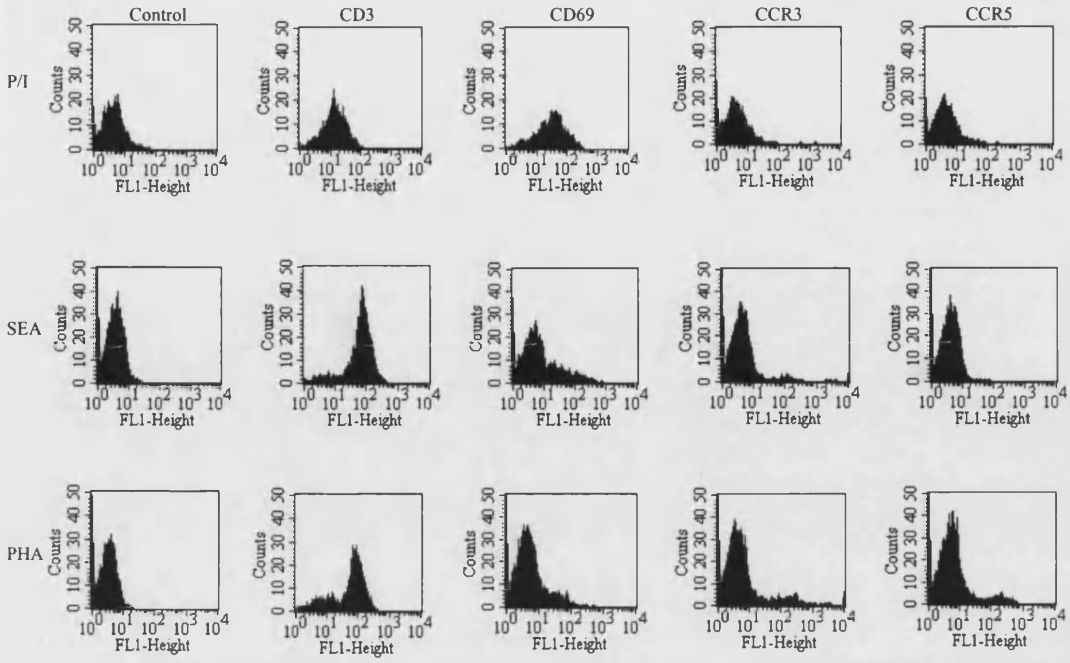


Figure 6.21: Surface Antigen Expression on T cell Blasts.
PBMCs were purified from normal blood and stimulated with PHA (2μg/ml), 5ng/ml PDBu/1μM ionomycin of SEA (10ng/ml). After six days cells were taken for co-culture with D3 THP-1 cells and also assessed for surface antigen expression using FACS analysis. The T cell receptor CD3, activation marker CD69 and chemokine receptors CCR3 and CCR5 were measured in order to give an idea of T cell purity, activation and possible helper family phenotype.

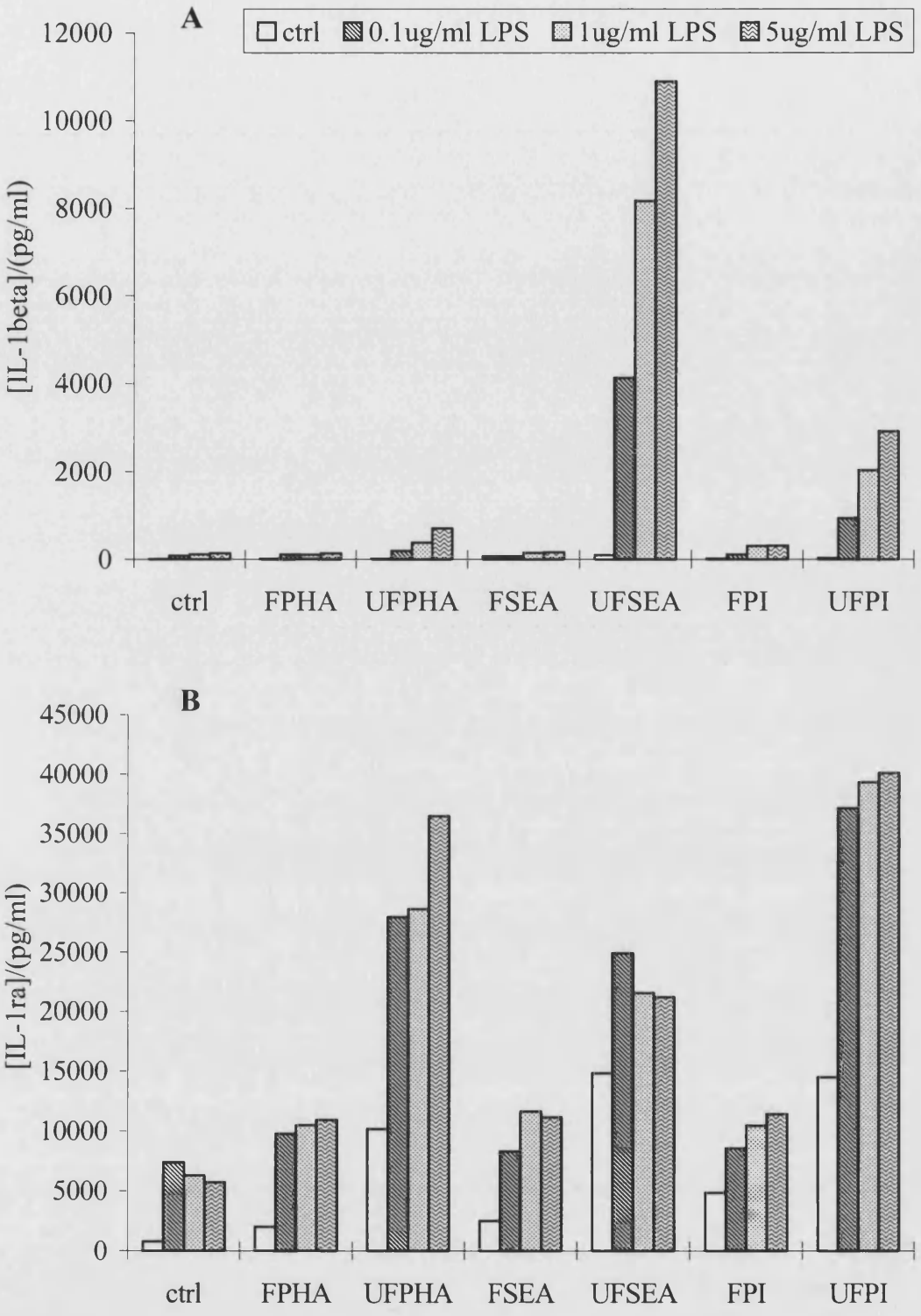


Figure 6.22: Effect of Fixation on Day Six PBMC Blast Driven THP-1 Cells.
Panel A: IL-1β release from D3 THP-1 cells stimulated with fixed/unfixed T cell blasts and various concentrations of LPS (n=1). Fixed (F) and unfixed (UF) blasts were derived from PHA (2μg/ml), SEA (10ng/ml) and PDBu (5ng/ml)/ionomycin (1μM) stimulated PBMCs (n=1).
Panel B: Parallel IL-1ra release from same supernatants.

6.2.11: Effect of PI3–K Inhibition on the Balance Between IL-1β and IL-1ra Production

IgG induced IL-1ra production is reported to involve PI3–K activity. The PI3–K inhibitor LY294002 (Sigma) (460) was used to challenge PBMC and D3 THP-1 responses to plastic adhered IgG, LPS and activated J16 cells.

Unfortunately, these data was not completed to statistical significance but does present clear trends that are worthy of note. The first experiment, displayed in **figure 6.23**, shows the effect of LY294002 (LY) on PBMC responses to LPS. As can be seen from the top panel, LY has no effect on LPS induced IL-1β. In contrast, control and LPS induced IL-1ra production is sensitive to LY in a concentration dependent manner.

Using activated J16 cells to drive PBMCs a weak induction of IL-1β was seen as well as a strong induction of IL-1ra (see **figure 6.24**). LY augmented IL-1β production but inhibited IL-1ra production in a concentration dependent manner.

PBMC responses to IgG were also affected by LY, as can be seen in **figure 6.25**. The IL-1β response to IgG was very small and LY showed inconsistent effects but with no obvious inhibition. In contrast the strong induction of IL-1ra, using IgG, was potently blocked by LY, with almost total inhibition seen with 10μM LY.

In D3 THP-1 cells LY was used to challenge IgG, J16, LPS and T cell blast induced IL-1ra production. As can be seen from **figures 6.26** and **6.27**, similarly to PBMC responses, LY inhibited IL-1ra production induced by LPS stimulation, Fc receptor aggregation or T cell co-culture.

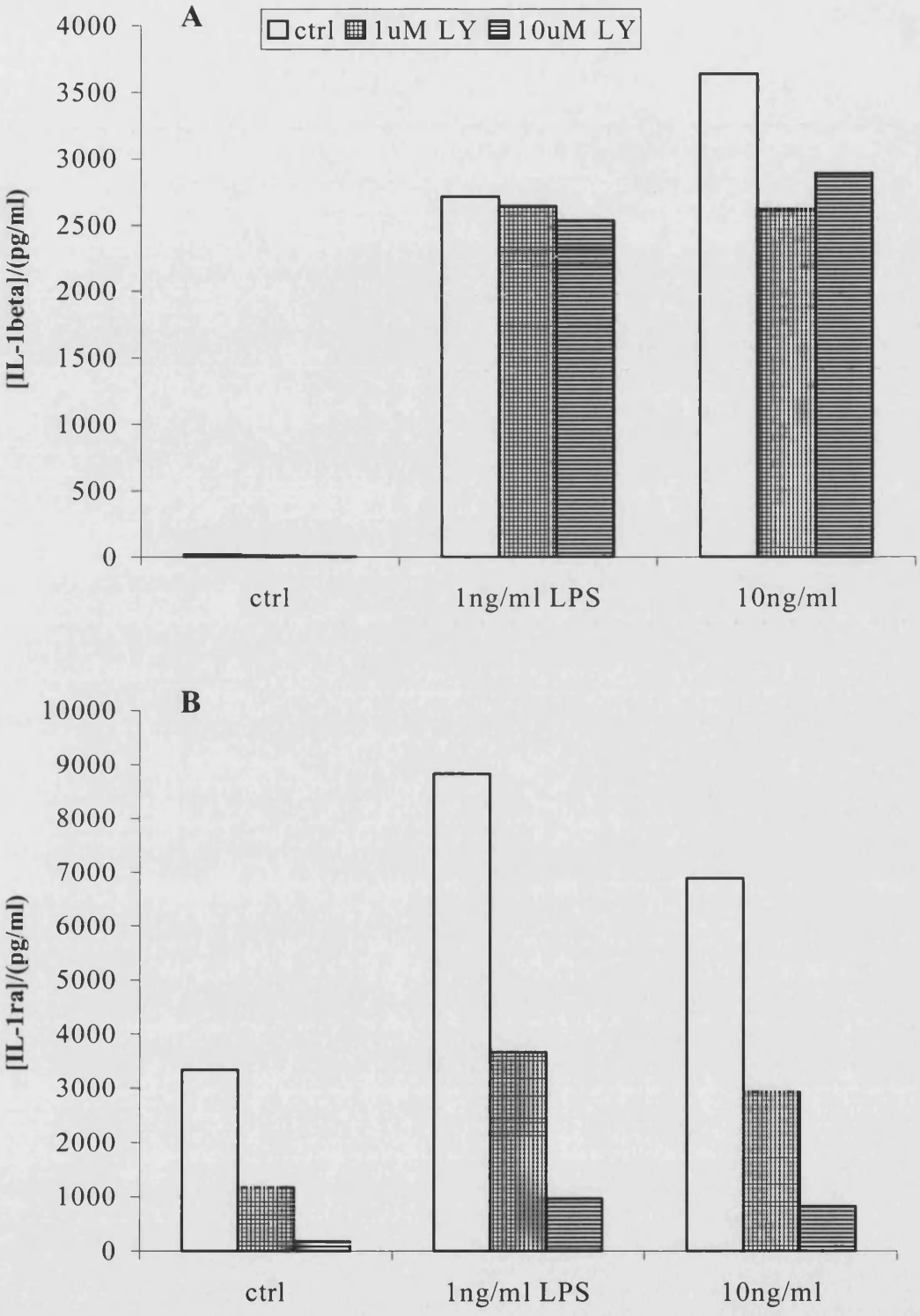


Figure 6.23: Effect of PI3-Kinase Inhibition on LPS Stimulated Cytokine Release from PBMCs.

Panel A: IL-1β release from PBMCs stimulated with LPS. In test samples the PBMCs were pre-treated with the PI3–K inhibitor LY294002 (LY) for 30 minutes prior to stimulation. Supernatants were harvested after 48 hours of culture (n=1).

Panel B: Parallel IL-1ra content of same supernatants.

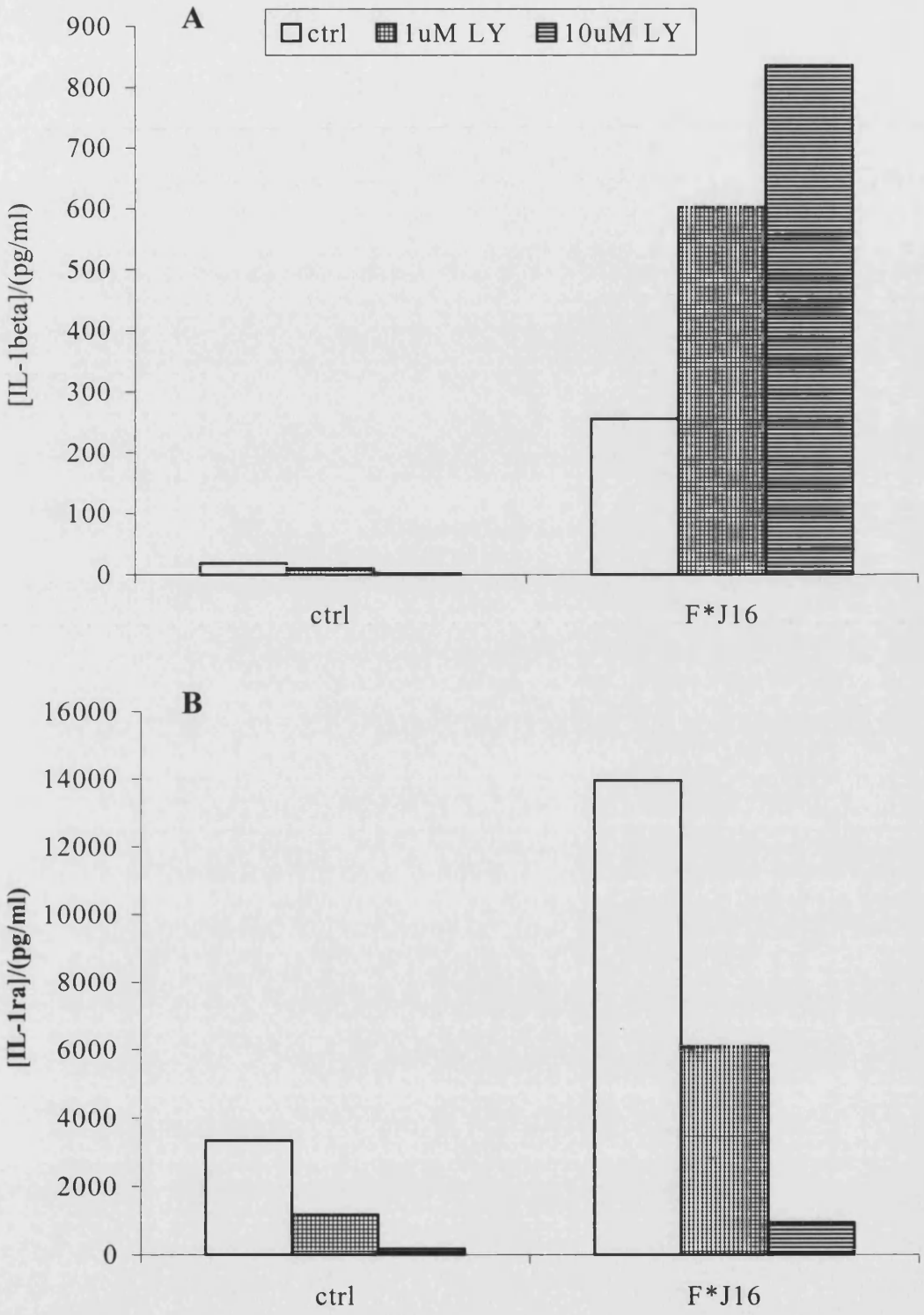


Figure 6.24: Effect of PI3-Kinase Inhibition on Activated J16 Stimulated Cytokine Release from PBMCs.

Panel A: IL-1β release from PBMCs stimulated with fixed activated J16s (F*J16). In test samples the PBMCs were pre-treated with the PI3–K inhibitor LY294002 (LY) for 30 minutes prior to stimulation. Supernatants were harvested after 48 hours of culture (n=1).

Panel B: Parallel IL-1ra content of same supernatants.

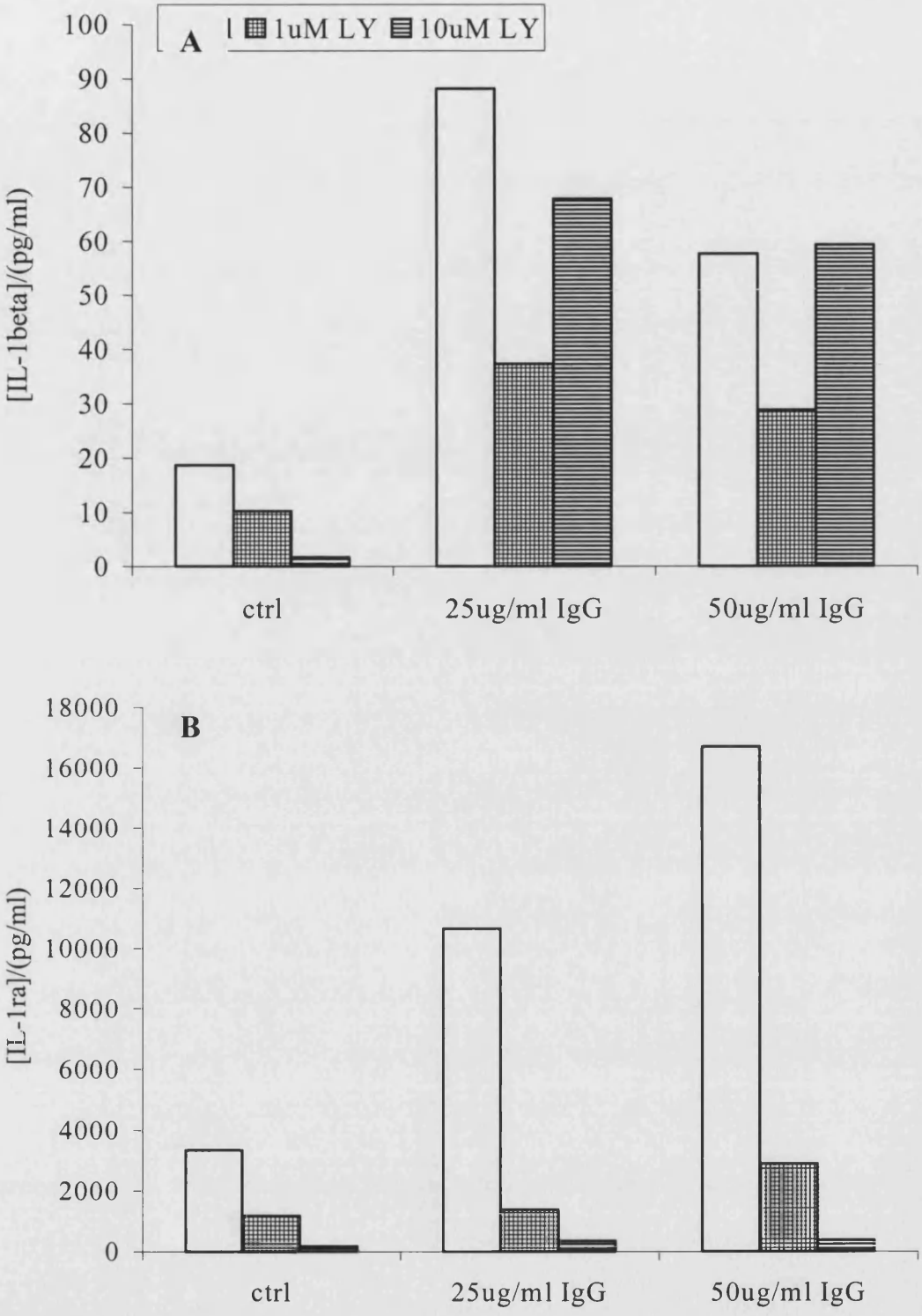


Figure 6.25: Effect of PI3-Kinase Inhibition on IgG Stimulated Cytokine Release from PBMCs.

Panel A: IL-1β release from PBMCs stimulated with plastic bound IgG. In test samples the PBMCs were pre-treated with the PI3–K inhibitor LY294002 (LY) for 30 minutes prior to stimulation. Supernatants were harvested after 48 hours of culture (n=1).

Panel B: Parallel IL-1ra content of same supernatants.

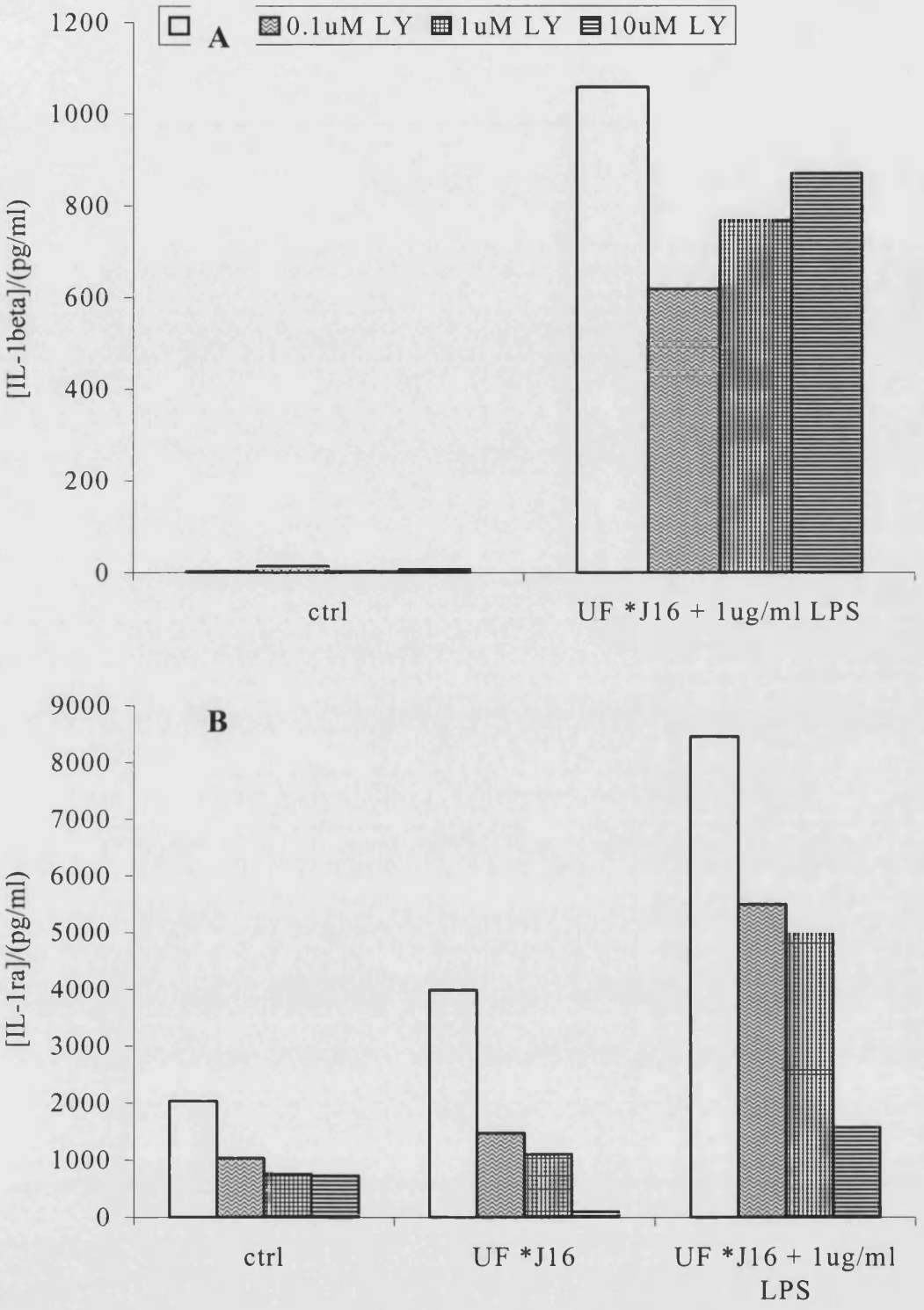


Figure 6.26: Effect of PI3-Kinase Inhibition on J16 and LPS Stimulated Cytokine Release from D3 THP-1 cells.

Panel A: IL-1β release from D3 THP-1 cells stimulated with LPS and unfixed activated J16s. D3 THP-1 cells were pre-treated with the PI3–K inhibitor LY294002 (LY) for 30 minutes prior to stimulation. Supernatants were harvested after 48 hours of culture (n=1).

Panel B: Parallel IL-1ra content of same supernatants and also form D3 THP-1 cells stimulated with unfixed activated J16s alone.

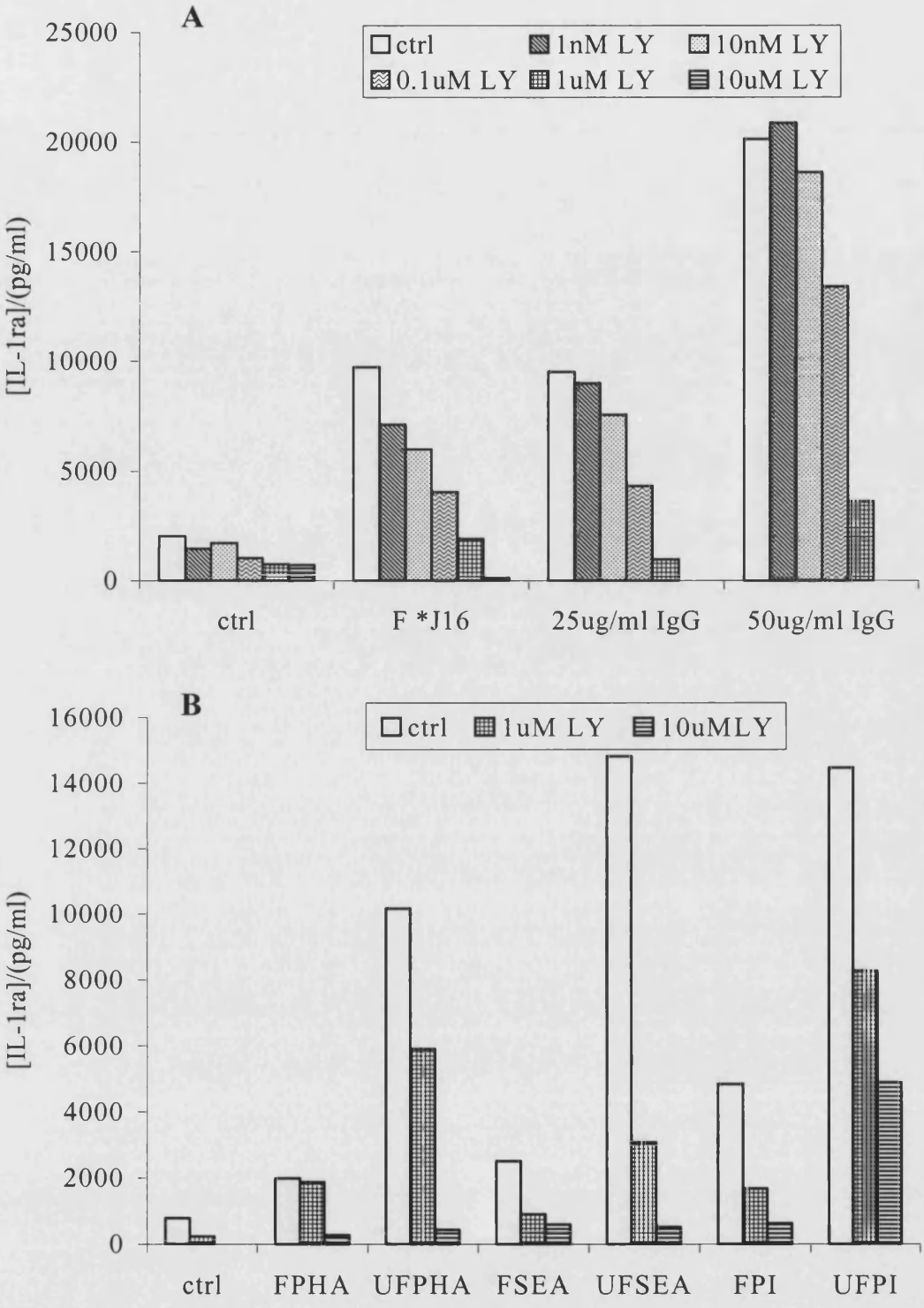


Figure 6.27: Effect of PI3-Kinase Inhibition on D3 THP-1 IL-1ra Production.
Panel A: IL-1ra release from D3 THP-1 cells stimulated with fixed activated J16s or plastic bound IgG.
Panel B: IL-1ra release from D3 THP-1 cells stimulated with fixed/unfixed T cell blasts. Fixed (F) and unfixed (UF) blasts were derived from PHA (2μg/ml), SEA (10ng/ml) and PDBu (5ng/ml)/ionomycin (1μM) stimulated PBMCs.
D3 THP-1 cells were pre-treated with the LY294002 (LY) for 30 minutes prior to stimulation. Supernatants were harvested after 48 hours of culture (n=1).

6.2.12: Effect of Ouabain on PBMC Responses to J16 Co-culture

Having established the conditions for co-culture it was hoped to assess the effect of ouabain on T cell induced IL-1β and IL-1ra responses. Unfortunately, due to time constraints, the following experiment has only been carried out twice so far.

In LPS stimulated PBMCs ouabain has been shown to dramatically induce a synergistic IL-1β response, and inhibit IL-1ra production. In the following experiments ouabain treated PBMCs were co-cultured with fixed and unfixed activated and resting J16 cells and the supernatants were analysed after forty-eight hours of culture. This time, however, slightly lower concentrations of ouabain were used (1nM and 10nM) in order to avoid the complication of any toxic effects. With LPS stimulation, 10nM ouabain had been shown to augment the IL-1β response and inhibit the IL-1ra response. The data for co-culture responses are displayed in **figure 6.28** and show the production of IL-1β in panel A and IL-1ra in panel B. In these experiments J16 cells, especially unfixed J16 cells induced a large increase in IL-1β production. Interestingly this induction of IL-1β was not dependent upon activation of the J16 cells. Under control conditions ouabain had little effect on IL-1β production but in the unfixed J16 groups a potent synergistic effect on IL-1β production was observed. Particularly affected was the response to unfixed activated J16 cells. Unfortunately in the parallel IL-1ra studies the J16 cells did not induce IL-1ra production from the PBMCs and as a result it is difficult to assess any effects of ouabain. In all groups studied, however, it can be seen that ouabain had a mild concentration dependent inhibitory effect on IL-1ra release.

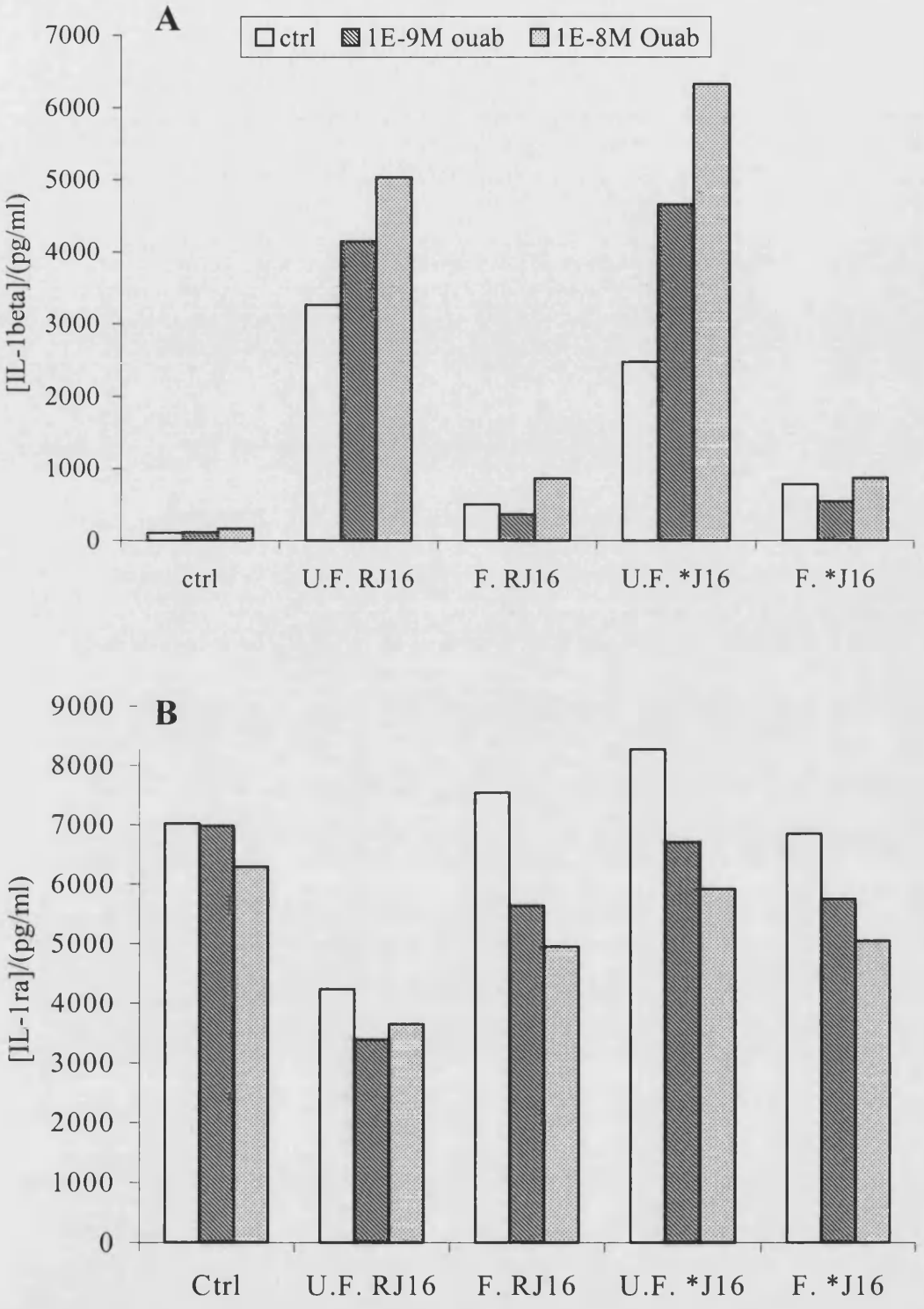


Figure 6.28: Effect of Ouabain on PBMC Responses to J16 Co-culture.
Panel A: IL-1β release from PBMCs stimulated with fixed (F) / unfixed (UF) activated (*) / resting (R) J16s.
Panel B: Parallel IL-1ra content of same supernatants.
PBMCs were pre-treated with the ouabain for 30 minutes prior to stimulation. Supernatants were harvested after 48 hours of culture (n=2).

6.3: SUMMARY

The aim of this chapter was to define conditions for improved study of the modulation of the balance between IL-1β and IL-1ra production from monocytes. It was hoped that, in determining stimuli which selectively modulated IL-1β or IL-1ra production, a model system could be developed in which to study the effect of modulatory compounds such as ouabain or anti-inflammatory drugs. The predominant system of interest was that of T cell co-culture, where activated or resting T cells can be used to stimulate the production of monocyte cytokines. This model has been described by Vey et al. (352) but it was hoped that we could expand it to explore the responses of rheumatoid PBMCs. By defining a model monocytic response it would be possible to measure the ability of T cells from different patient groups to stimulate IL-1β and IL-1ra release. Similarly, by defining a model T cell it would be possible to measure the responsiveness of patient monocytes to a T cell derived stimulus.

Initial experiments detailed here sought to define model monocytic IL-1β and IL-1ra responses. The THP-1 cell line was used to explore IL-1β and IL-1ra responses to LPS and IgG. Upon differentiation with $1,25(OH)_2$-vitamin D3 (D3) expression of CD14 was induced in THP-1 cells but expression of CD32 and CD64 remained at resting levels. In agreement with this, it was observed that LPS induced IL-1β production in THP-1 cells was D3 dependent but IgG induced IL-1ra production was not. Consequently, D3 differentiated THP-1 cells were considered to be a suitable model monocyte as they responded to control stimuli for both IL-1β and IL-1ra production.

PBMC and purified blood monocytes were also compared for their responsiveness to LPS and IgG stimulation. It was expected that as IL-1β and IL-1ra are monocyte-derived cytokines their production would be greatly increased in a purified monocyte preparation. In contrast, however, considering that the monocyte preparations routinely contained ten-fold more monocytes, little difference in cytokine production was observed. LPS stimulated IL-1β levels were very similar whereas IgG induced IL-1ra production was about two-fold higher in the purified monocyte preparation. Interestingly, in both PBMCs and monocytes, IgG was a very poor inducer of IL-1β and LPS was a very poor inducer of IL-1ra. IL-1ra levels were very high in control

conditions, perhaps due to adherence activation. In contrast, THP-1 cells, which do not adhere to plastic responded to LPS with both IL-1β and IL-1ra production, although IL-1β production is dependent upon the differentiation state of the THP-1 cells. However, in THP-1 cells IgG was also a poor inducer of IL-1β.

In light of the time-course of IL-1ra production observed in chapter 4 an extended study was made in these experiments. IL-1ra production was seen to increase dramatically over seventy-two hours but after twenty-four hours IL-1β levels only climbed slowly. As a result, from this point onwards all comparisons between IL-1β and IL-1ra release were made after a forty-eight hour culture period.

Before a T cell-monocyte co-culture system could be established basic parameters of T cell activation had to be defined. T cells from healthy volunteers and rheumatoid patients, as well as J16 cells, were tested for proliferative and surface activation marker responses to various stimuli. Anti-CD3 mAb, PDBu and ionomycin were used in combination with CHO-CD80 co-stimulation. Anti-CD3 stimulation and to a similar extent PDBu synergised with CD80 and ionomycin to induce potent proliferative responses in T cells. PDBu and ionomycin induced proliferation was also observed to correlate with surface CD69 expression. In J16 cells, however, none of the stimuli studied were able to modulate the already high proliferation rate. PDBu and ionomycin did however induce a concentration dependent increase in surface CD69 expression comparative to that seen in normal T cells. As a result of these studies 5ng/ml PDBu and 1μM ionomycin was used to induce J16 activation for monocyte co-culture and surface CD69 expression was monitored to measure the duration of the activated state. As T cell responses to the various stimuli were much subtler than those of J16 cells T cells activated with a broader range of stimuli were considered for co-culture.

Interestingly, when observing the proliferative responses of rheumatoid T cells it was found that there was a marked impairment in anti-CD3 responses. Responses to PDBu, which activates signalling cascades down-stream of the TCR, were comparable between rheumatoid and normal T cells as were the synergistic responses to PDBu and CD80. When assessing surface marker expression in resting T cells it was seen that CD3 expression was no different in rheumatoid T cells. Consequently, it can be concluded that CD3 in rheumatoid T cells does not transduce signals as effectively as that in normal T cells upon antibody induced aggregation.

When fixed J16 cells were used in co-culture to deliver a membrane bound stimulus, it was found that IL-1β and IL-1ra production was induced in THP-1 cells. The production of IL-1β and to a similar extent IL-1ra was dependent upon J16 activation with PDBu and ionomycin. In PBMCs and purified monocytes resting and activated fixed J16 cells induced IL-1β production but had less of an effect on IL-1ra production. Monocyte and PBMC basal production of IL-1ra was so high that it is possible that any stimulatory effects would not be as dramatically noticed as they were in THP-1 cells. Nevertheless, activated J16 cells produced a mild induction of IL-1ra in monocytes and a slightly more pronounced effect in PBMCs.

It is worthy of note that the magnitude of the IL-1ra response to J16 stimulation (resting or activated) was much greater than that of IL-1β in terms of the concentration of protein secreted.

Preliminary studies using activated T cells, which were then co-cultured with their autologous monocytes, showed a similar profile of activation dependent induction of IL-1β and IL-1ra. Due to the complexity of this experiment and the amount of blood needed to be taken from a patient to allow the separate preparation of enough T cells and monocytes for co-culture, only two experiments were carried out. These results did, however, confirm that it was possible to differentiate between the activation state of normal T cells based upon their ability to induce monocyte cytokine production. These data supported the possibility of measuring monocyte responses to rheumatoid T cells and comparing them to normal healthy T cells.

To increase the understanding of the THP-1 response to J16 cells various attempts were made to modulate the IL-1ra and IL-1β responses. Although activated J16 cells induced the secretion of both IL-1β and IL-1ra the magnitude of the IL-1ra response dominated. Consequently, LPS was used to prime the THP-1 cells in order to see if there would be a shift in the balance between IL-1β and IL-1ra production. In parallel experiments IgG was used to prime an IL-1ra response. It was found that LPS synergised greatly with activated J16 cells to induce IL-1β production but had little effect on IL-1ra production. In contrast, IgG synergised with resting and activated J16 cells to induce both IL-1β and IL-1ra. The levels of IL-1β induced by IgG and J16 stimulation were however ten-fold lower than those observed with LPS and J16 cells. This dramatic synergy between J16 and LPS stimulation on THP-1 production

of IL-1β was explored further to assess the role of the J16 cells. The signal coming from a fixed J16 was supposed to be entirely due to membrane bound interactions but to observe whether soluble mediators from the J16 cells could have any effect on THP-1 responses both fixed and unfixed J16 cells were used. In these studies unfixed J16 cells synergised more greatly with LPS to induce IL-1β secretion than did fixed J16 cells. These data suggested that LPS could not only synergise with membrane-derived signals but also soluble mediator signals from J16 cells. Interestingly, fixed J16 cells induced greater IL-1ra production than unfixed J16 cells suggesting the possibility that membrane bound signals from J16 cells favour the induction of IL-1ra whereas soluble mediators favour the induction of IL-1β.

In PBMCs a similar story was observed where unfixed J16 cells synergised greatly with LPS to induce IL-1β production but fixed J16 cells had only a modest effect. In this experiment, however, IL-1ra production was not induced consistently by any of the stimuli so no synergistic effects could be described. In attempts to study T cell driven IL-1ra experiments were established to examine the effect of LPS on resting and activated normal T cell stimulated THP-1 cells. Unfortunately, however, due to inadequacies in the T cell purification protocol any contamination of the T cell preparation with monocytes dramatically skewed the levels of IL-1β produced in response to LPS co-stimulation. As the THP-1 cells needed high levels of LPS to induce IL-1β and IL-1ra responses if there were any blood monocytes in the T cell preparation they produced so much IL-1β that the THP-1 responses were swamped. As a result, to avoid this problem different T cell blasting protocols were tested to produce a population of pure activated T cells from blood PBMCs. Super antigen (SEA) and PDBu/Ionomycin were used to activate PBMCs. On days three, five and seven after stimulation T cells were taken, assessed for purity and activation via surface marker expression and then used for co-culture with THP-1 cells. All experiments showed that the T cell blasts could induce IL-1β and IL-1ra production and that there was synergy between LPS and T cell driven IL-1β. LPS and T cell stimulation had an additive effect on IL-1ra production. The synergy between LPS and T cell driven IL-1β was most marked using day seven SEA blasts. These T cells did not appear different according to surface phenotype but the induction of IL-1β was dramatic. When unfixed T cells were compared to fixed T cells for their ability to synergise with LPS at inducing IL-1β production it was again found that fixation

inhibited the IL-1β response. It appears that soluble, T cell derived mediators are dominant in the synergy between LPS and T cell stimulated IL-1β production.

Interestingly, in all experiments there was a correlation between CD69 expression and the induction of IL-1ra production (R=0.727, P=0.024) but not IL-1β production (R = -0.383, P = 0.323).

Unfortunately, at this point in the co-culture studies there was insufficient time to further explore the phenomenon that have been described here. Due to the award of a Basic Science Initiative grant from Pharmacia and Upjohn further studies were dedicated to the investigation of whether Sulphasalazine could modulate T cell-monocyte co-culture responses. In some complementary studies, however, the PI3–Kinase inhibitor LY294002 (LY) was used to challenge the co-culture model. As PI3–Kinase is known to be involved in FcR signalling it was suggested that LY may well modulate IgG induced responses of PBMCs and THP-1 cells. To test whether T cell derived signals may utilise similar signalling pathways responses were challenged with various concentrations of LY, and IL-1β and IL-1ra production was monitored. In PBMCs LY inhibited LPS induced IL-1ra, fixed activated J16 induced IL-1ra and IgG induced IL-1ra in a concentration dependent manner. The induction of IL-1β by all of these stimuli was unaffected by LY. Similarly, in THP-1 cells LY inhibited LPS, IgG, J16 and T cell blast induced IL-1ra but not IL-1β.

The effect of Na^+/K^+-ATPase modulation on J16 driven PBMC cytokine production was also studied in two preliminary experiments. The results clearly showed that ouabain synergised with J16 cells to induce IL-1β production but inhibited IL-1ra production. Stimulation with unfixed activated J16 cells synergised most greatly with ouabain suggesting the possibility that ouabain may modulate the production of J16 derived soluble mediators.

CHAPTER 7

Generation of a CD69 Transfectant for Monocyte Activation

7.1: INTRODUCTION

The studies described in chapter six clearly demonstrate that T cells are capable of modulating the production of IL-1β and IL-1ra from monocytes. The use of T cell fixation differentiated between signals coming solely from T cell-monocyte membrane interactions and those resulting from the combined effect of T cell derived cytokines and membrane interactions. In particular, fixed activated J16 cells strongly induced IL-1ra production from the THP-1 cell line in the absence of a significant IL-1β response. Conversely, unfixed activated J16 cells were able to synergise with LPS to dramatically induce IL-1β production whilst attenuating the IL-1ra response. This evidence suggests the possibility that T cell derived soluble mediators and membrane antigens differentially regulate monocyte cytokine production. Evidence from recent literature has implied the role of several T cell surface molecules in the regulation of monocyte responses in co-culture. McAllister and Ellis (1996) have shown inhibition of T cell driven peripheral blood monocyte IL-1β production using antibodies that block CD2 interactions with CD58 (368). In THP-1 cells, however, responses to T cell stimulation have been inhibited using anti-CD69, CD11a, CD11b and CD11c (352,354). Using freshly isolated T cells anti-CD69 mAb in particular have been shown to inhibit 70% of monocyte IL-1β production. From these data it is apparent that no single T cell surface molecule is responsible for the induction of monocyte responses. It is likely that, as well as critical T cell signalling molecules, a complex interaction of adhesion molecules is also crucial for monocyte activation. The rapidly inducible nature of the activation dependent T cell membrane molecules combined with the anti-CD69 mAb data strongly suggested CD69 as a likely candidate for further investigation. The evidence presented in chapter 6 also supports a role for CD69 as the ability of activated peripheral blood T cells to induce D3 THP-1 IL-1ra release correlated with CD69 expression. In an attempt to achieve the cleanest system for analysis of CD69 dependent monocyte activation it was decided to clone human CD69 in order to generate a transfected cell line. By ensuring that CD69 is the only T cell antigen expressed on the transfectant, clear data as to the role of CD69 in monocyte activation could be constructed.

7.2: METHODS

The details of all protocols used in the cloning and transfection of human CD69 are detailed in Chapter 2, section 2.9. Any practical considerations relevant to the specific enzymes, cloning techniques and reagents used will be detailed here, along with the results.

7.3: RESULTS

7.3.1: Production of cDNA

Human CD69 is rapidly expressed in J16 cells upon activation with various stimuli. In this study, CD69 surface expression, as determined by antibody staining using flow cytometry, was induced with the phorbol ester PDBu and the calcium ionophore ionomycin. CD69 expression was monitored over a twenty-four hour time-course and as can be seen from **figure 7.1** was readily measurable within two hours. By eight hours nearly the entire population of cells became CD69 positive. Using this evidence PDBu and ionomycin were tested at various concentrations to activate J16 cells to induce CD69 expression. As can be seen in **figure 7.2**, PDBU and ionomycin induced CD69 expression in an additive and concentration dependent manner. Consequently, 5ng/ml PDBu combined with 1μM ionomycin was used to induce CD69 mRNA production. Within two hours of stimulation the percentage of cells expressing CD69 was already high, at 88%. The level of protein expression was quite low, however, as can be determined by the magnitude of the shift in FL-1 fluorescence. Consequently, a two-hour time-point was used for mRNA harvesting.

Messenger RNA was prepared as described in **method 2.8.1** and reverse transcribed into cDNA as described in **method 2.8.3**.

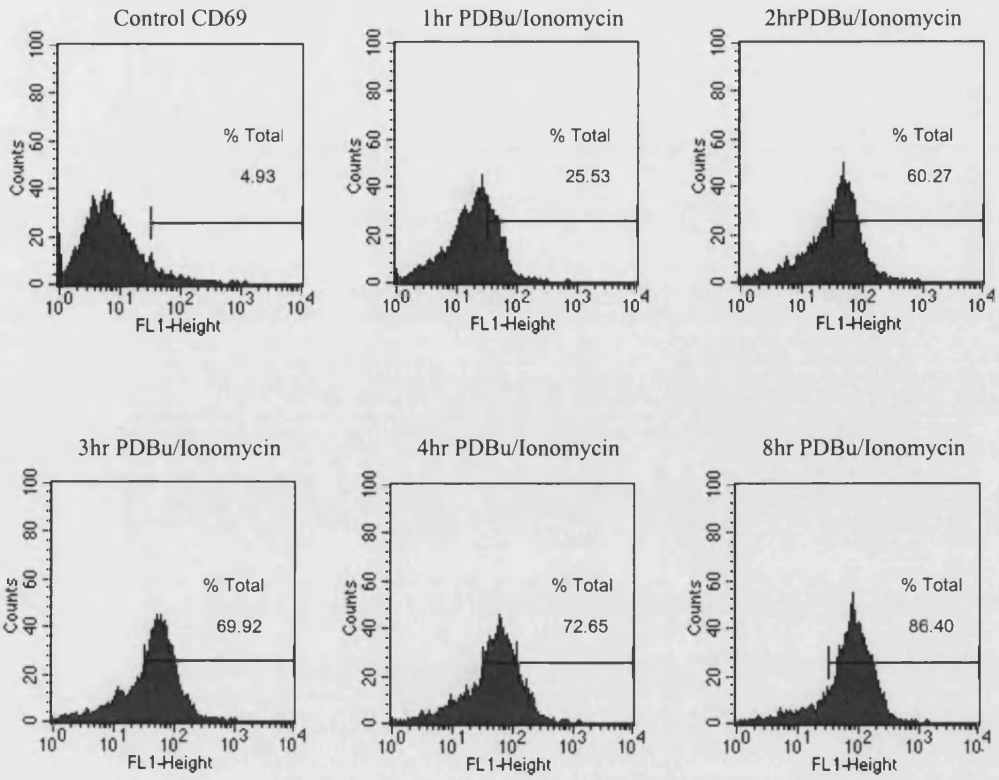
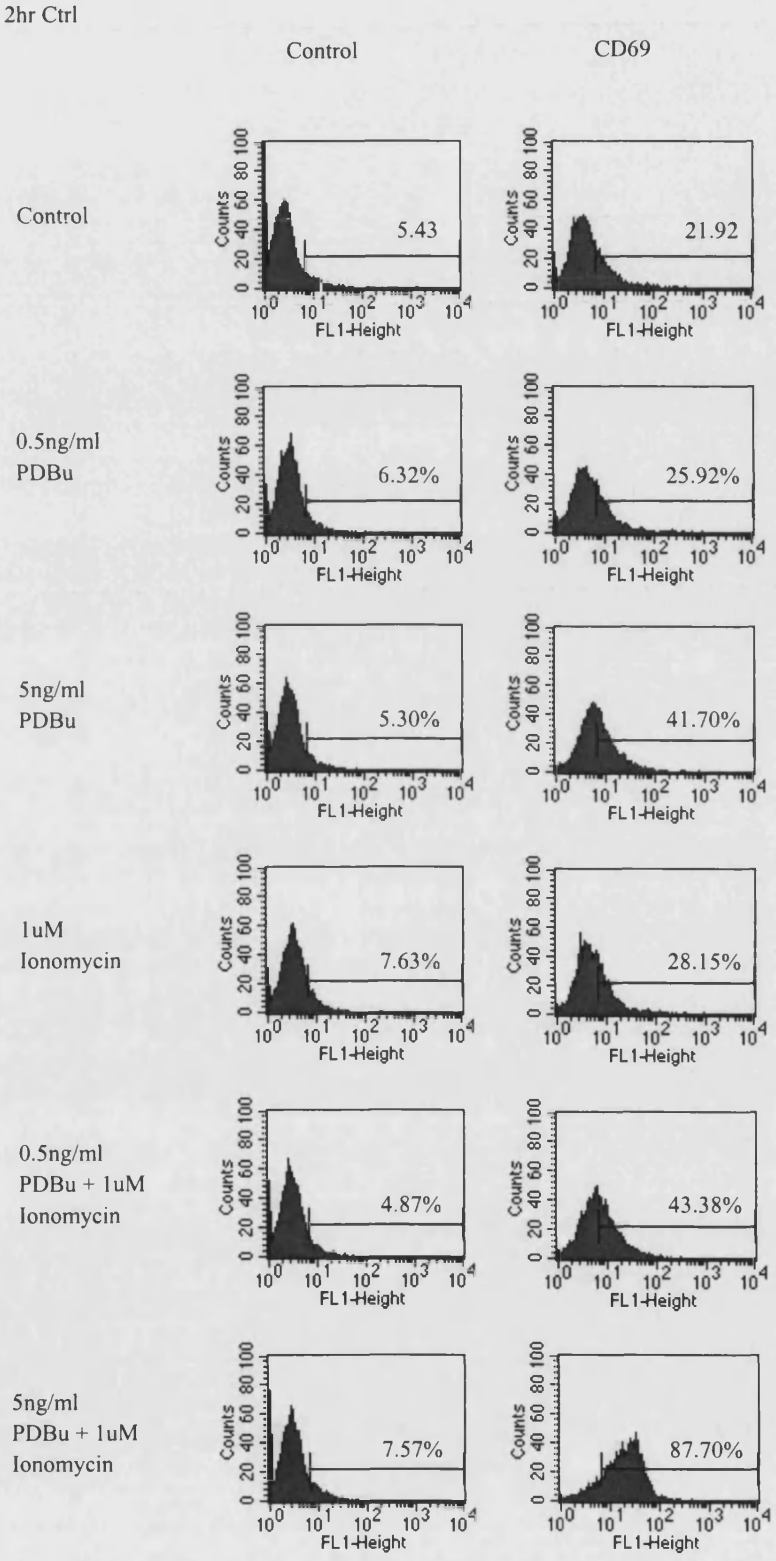


Figure 7.1: Time-course of CD69 expression induced by 5ng/ml PDBu + 1μM Ionomycin in J16 cells.

Figure 7.2: Induction of CD69 protein expression in J16 cells after two hours of activation.



7.3.2: CD69 Sequence and Primer Design

The gene for CD69 is 1662 bases long, of which section 56 to 682 codes for the Type II transmembrane protein. Using this sequence information, shown below, CD69 forward and reverse primers were designed.

Sequence Range: 56 to 700

GGA ATC TTG AGA ATA AAG ATG AGC TCT GAA AAT TGT TTC GTA
Gly Ile Leu Arg Ile Lys Met Ser Ser Glu Asn Cys Phe Val>

GCA GAG AAC AGC TCT TTG CAT CCG GAG AGT GGA CAA GAA AAT
Ala Glu Asn Ser Ser Leu His Pro Glu Ser Gly Gln Glu Asn>

GAT GCC ACC AGT CCC CAT TTC TCA ACA CGT CAT GAA GGG TCC
Asp Ala Thr Ser Pro His Phe Ser Thr Arg His Glu Gly Ser>

TTC CAA GTT CCT GTC CTG TGT GCT GTA ATG AAT GTG GTC TTC
Phe Gln Val Pro Val Leu Cys Ala Val Met Asn Val Val Phe>

ATC ACC ATT TTA ATC ATA GCT CTC ATT GCC TTA TCA GTG GGC
Ile Thr Ile Leu Ile Ile Ala Leu Ile Ala Leu Ser Val Gly>

CAA TAC AAT TGT CCA GGC CAA TAC ACA TTC TCA ATG CCA TCA
Gln Tyr Asn Cys Pro Gly Gln Tyr Thr Phe Ser Met Pro Ser>

GAC AGC CAT GTT TCT TCA TGC TCT GAG GAC TGG GTT GGC TAC
Asp Ser His Val Ser Ser Cys Ser Glu Asp Trp Val Gly Tyr>

CAG AGG AAA TGC TAC TTT ATT TCT ACT GTG AAG AGG AGC TGG
Gln Arg Lys Cys Tyr Phe Ile Ser Thr Val Lys Arg Ser Trp>

ACT TCA GCC CAA AAT GCT TGT TCT GAA CAT GGT GCT ACT CTT
Thr Ser Ala Gln Asn Ala Cys Ser Glu His Gly Ala Thr Leu>

GCT GTC ATT GAT TCT GAA AAG GAC ATG AAC TTT CTA AAA CGA
Ala Val Ile Asp Ser Glu Lys Asp Met Asn Phe Leu Lys Arg>

TAC GCA GGT AGA GAG GAA CAC TGG GTT GGA CTG AAA AAG GAA
Tyr Ala Gly Arg Glu Glu His Trp Val Gly Leu Lys Lys Glu>

CCT GGT CAC CCA TGG AAG TGG TCA AAT GGC AAA GAA TTT AAC
Pro Gly His Pro Trp Lys Trp Ser Asn Gly Lys Glu Phe Asn>

AAC TGG TTC AAC GTT ACA GGG TCT GAC AAG TGT GTT TTT CTG
Asn Trp Phe Asn Val Thr Gly Ser Asp Lys Cys Val Phe Leu>

AAA AAC ACA GAG GTC AGC AGC ATG GAA TGT GAG AAG AAT TTA
Lys Asn Thr Glu Val Ser Ser Met Glu Cys Glu Lys Asn Leu>

TAC TGG ATA TGT AAC AAA CCT TAC AAA TAA TAA GGA AAC GTG
Tyr Trp Ile Cys Asn Lys Pro Tyr Lys *** *** Gly Asn Val>

TTC ACT TAT TGA CTA
Phe Thr Tyr *** Leu>

The CD69 primers were tested against the sequence of CD69 using the MacVector program. The data below shows the primer sequence used, the physical properties of the primers and their specificity for the CD69 sequence.

Human CD69 Sequence Scanned: 56 to 682

CD69 Forward: 5' GGAATCTTGAGAATAAAGATGAGC 3'
length: 24, %GC: 37.5, Gs: 7, Cs: 2, ambiguous G or C: 0
Tm: 46.4 °C, (Of Primer itself)
1μg of primer is equivalent to 415.9 pmole of ends
Primer does not form Self 3'-dimer
Primer does not form Hairpin
Primer does not form Self Duplex
Primer binds at position 56 on the Top Strand (score 24)

CD69 Reverse: 5' GTTTCCTTATTATTTGTAAGG 3'
length: 21, %GC: 28.6, Gs: 4, Cs: 2, ambiguous G or C: 0
Tm: 36.5 °C, (Of Primer itself)
1ug of primer is equivalent to 440.6 pmole of ends
Primer does not form Self 3'-dimer
Primer does not form Hairpin
Primer does not form Self Duplex
Primer binds at position 682 on the Bottom Strand (score 21)

Product details:

Product: 627 bp (56-682)
Optimal annealing temp: 49.5,
pct G+C: 42.6 Tm: 76.3 °C

```
5' - G G A A T C T T G A G A A T A A A G A T G A G C- 3' (CD69 F)
     | | | | | | | | | | | | | | | | | | | | | | | | |
56 5'-G  G A A T C T T G A G A A T A A A G A T G A G C - 583bp
   3'-C  C T T A G A A C T C T T A T T T C T A C T C G

   C C T T A C A A A T A A T A A G G A A A C - 682
   G G A A T G T T T A T T A T T C C T T T G
   | | | | | | | | | | | | | | | | | | | | | | | |
3' G G A A T G T T T A T T A T T C C T T T G- 5' (CD69 R)
```

7.3.3: Production of CD69 cDNA

The cDNA prepared from PDBu/ionomycin stimulated J16 cells was used as a template for PCR amplification using the CD69 forward and reverse primers described above. An annealing temperature range of 49°C to 52°C was studied, based on the optimal temperature of 49.5°C recommended by the Mac Vector software. The 627 base pair product generated was visualised on a 1% Agarose gel which is shown in **figure 7.3**. Once optimal PCR conditions had been defined they were used to produce a CD69 cDNA fragment which was suitable for directional insertion into the pCR3.1-Uni vector (Invitrogen).

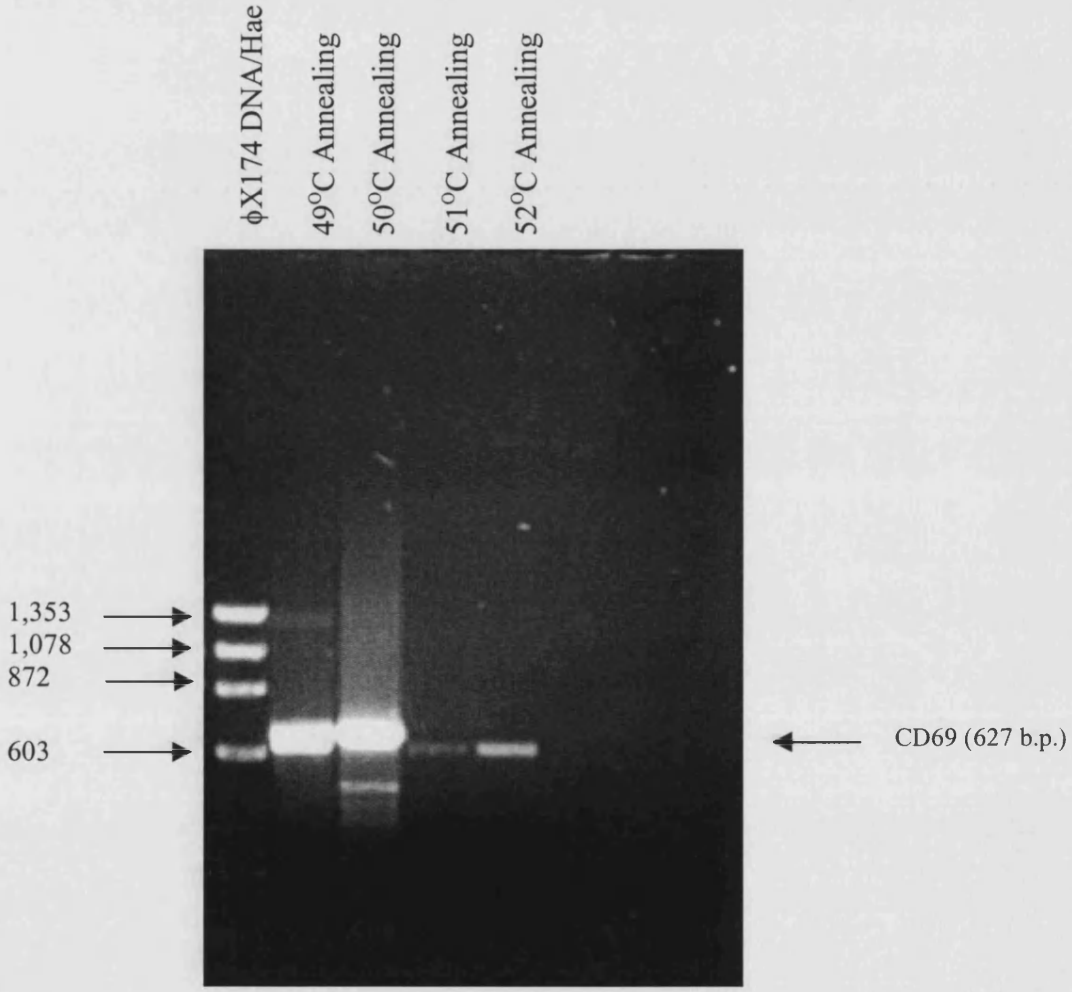


Figure 7.3: Effect of Annealing temperature on PCR amplification of CD69 cDNA.

7.3.4: Unidirectional Cloning

Unidirectional cloning exploits the nontemplate-dependent activity of *Taq* polymerase which adds a single deoxyadenosine (A) to the 3' ends of PCR products. The vector used in this study, pCR3.1-Uni, has single 3' deoxythymidine (T) residues that ligate readily to PCR products with A-overhangs. To achieve directional insertion of PCR products, however, the forward PCR primer is phosphorylated prior to amplification. The resulting PCR product is only phosphorylated at the 5' end, allowing ligation into the modified vector in only one orientation.

Attempts to insert CD69 cDNA in both orientations were made in order to produce a control clone that did not express CD69 protein. To achieve this the CD69 forward and reverse primers were phosphorylated and used in separate PCR reactions. To phosphorylate the primer, 0.5-1μg was incubated at 37°C for thirty minutes with 1μl 10X buffer, 1μl 10mM ATP, 1μl T4 polynucleotide kinase (10U/μl) and sterile water (up to 10μl). The phosphorylation reaction was stopped by incubation at 94°C for five minutes and then the primer was used immediately, with the complementary un-phosphorylated primer, to amplify CD69 for ligation.

Insertion of fresh uni-directional PCR product was carried out as described in section 2.9.7. The ligated vectors produced were then used immediately to transform TOP10F' cells as described in **method 2.8.9**.

The vector modification also rules out self-ligation in the absence of PCR fragment insertion improving the transformation efficiency.

7.3.5: Colony Screening for CD69 Insertion

Either side of the insertion point in the pCR3.2-Uni vector is a T7 promoter/priming site and a pCR3.1 reverse priming site. The primers for these sites allowed the transformed colonies to be screened for CD69 cDNA insertion via PCR amplification of the polylinker sequence. Bacteria were transferred from individual colonies to PCR reaction tubes and the DNA was amplified using the previously described reaction. **Figure 7.4** shows the results from eleven selected colonies compared to control CD69 PCR product. CD69 positive colonies yielded a PCR product equivalent in size to CD69 (627 bases) plus the polylinker site (approximately 145

bases). Lanes 3, 7, 8, 9 and 10 clearly display products of approximately 800bp which represent insertion of CD69 DNA.

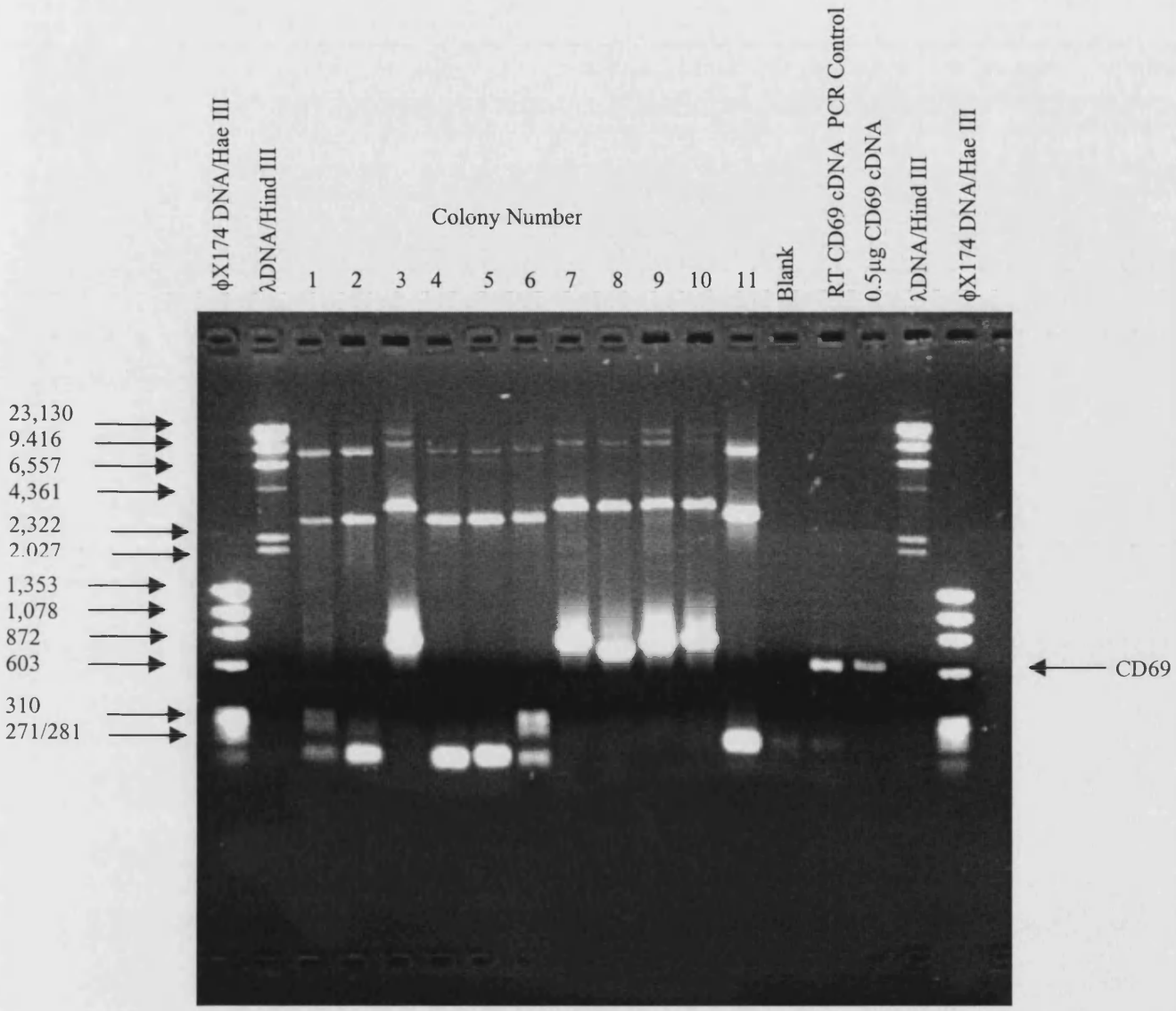


Figure7.4: PCR Screen of Transformed colonies.
Lanes one to five contain DNA from colonies selected form the CD69 reverse ligation reactions. Low molecular weight bands represent amplification of the polylinker region in the absence of CD69 insertion. In lane three the low molecular weight band is replaced with an intense band at approximately 800 bp which represents inserted CD69.
Lanes six to eleven represent colonies from CD69 forward reactions and clearly display CD69 insertion in lanes seven to ten.

7.3.6: Confirmation of CD69 DNA Orientation in pCR3.1 Vector

Even though a unidirectional cloning system was used, the positive clones identified above were tested for insert orientation by restriction mapping. DNA was prepared using the mini-prep system described in **method 2.8.10** and was digested with the enzymes Sac 1, Nco 1 and Bst X1. **Figures 7.5** and **7.6** show the restriction sites for these enzymes in the pCR3.1-uni vector, including CD69 DNA inserted in a forward and reverse reading direction respectively. The digestion fragment sizes produced are listed in **table 7.1**.

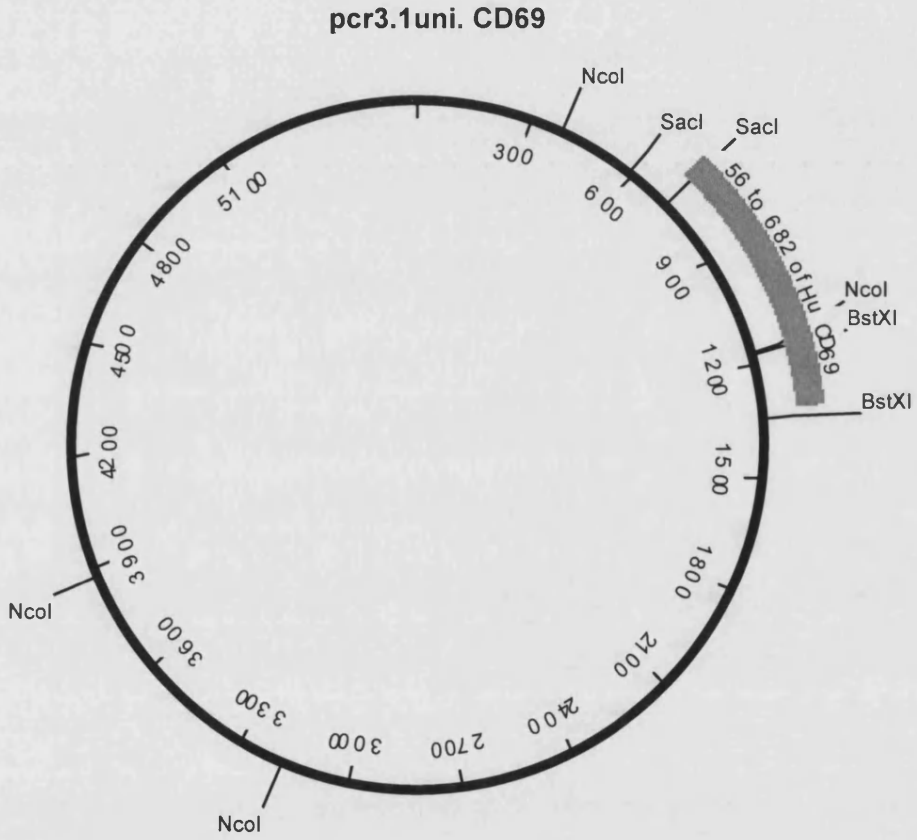


Figure 7.5: pCR3.1 (CD69F) Restriction map for Nco 1, Bst X1 and Sac 1.

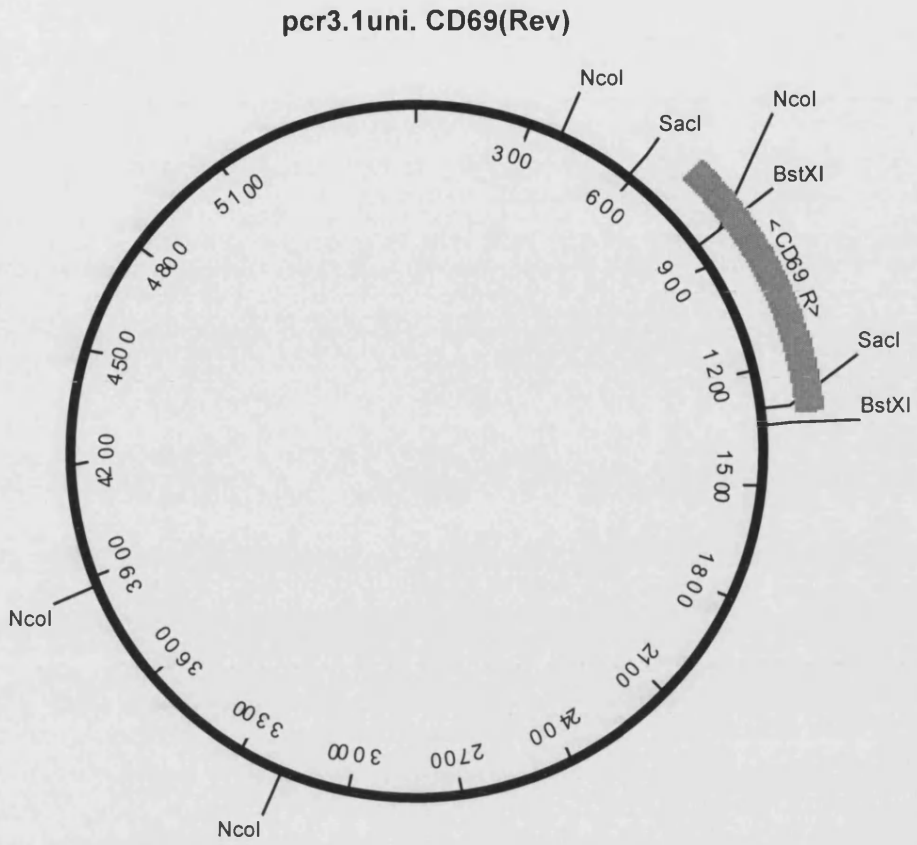


Figure 7.6: pCR3.1 (CD69R) Restriction map for Nco 1, Bst X1 and Sac 1

Enzyme	**CD69 Forward**		CD69 Reverse	
	Fragment Size	Cut From:To	Fragment Size	Cut From:To
Nco 1	2148	3876:385	2334	845:3178
	2013	1166:3178	2148	3876:385
	780	386:1165	697	3179:3875
	697	3179:3875	459	386:844
Sac 1	5513	719:593	4932	1300:593
	125	594:718	706	594:1299
BstX1	5467	1344:1172	5140	1344:845
	171	1173:1343	498	846:1343

Table 7.1: DNA fragment sizes for pCR3,1 containing CD69 DNA in a forward or reverse direction.

DNA from six of the transformed colonies was digested with each of the three restriction enzymes. Three of the CD69F insert positive colonies identified in **figure 7.4** (lanes 7-9) were used as were three CD69R colonies. Of the three CD69R

colonies only one had been identified in as being insert positive (lane 3) the other two were fresh mini-prep products from colonies transferred from new colonies. The restriction digests were resolved on a 1% agarose gel as displayed in **figure 7.7**.

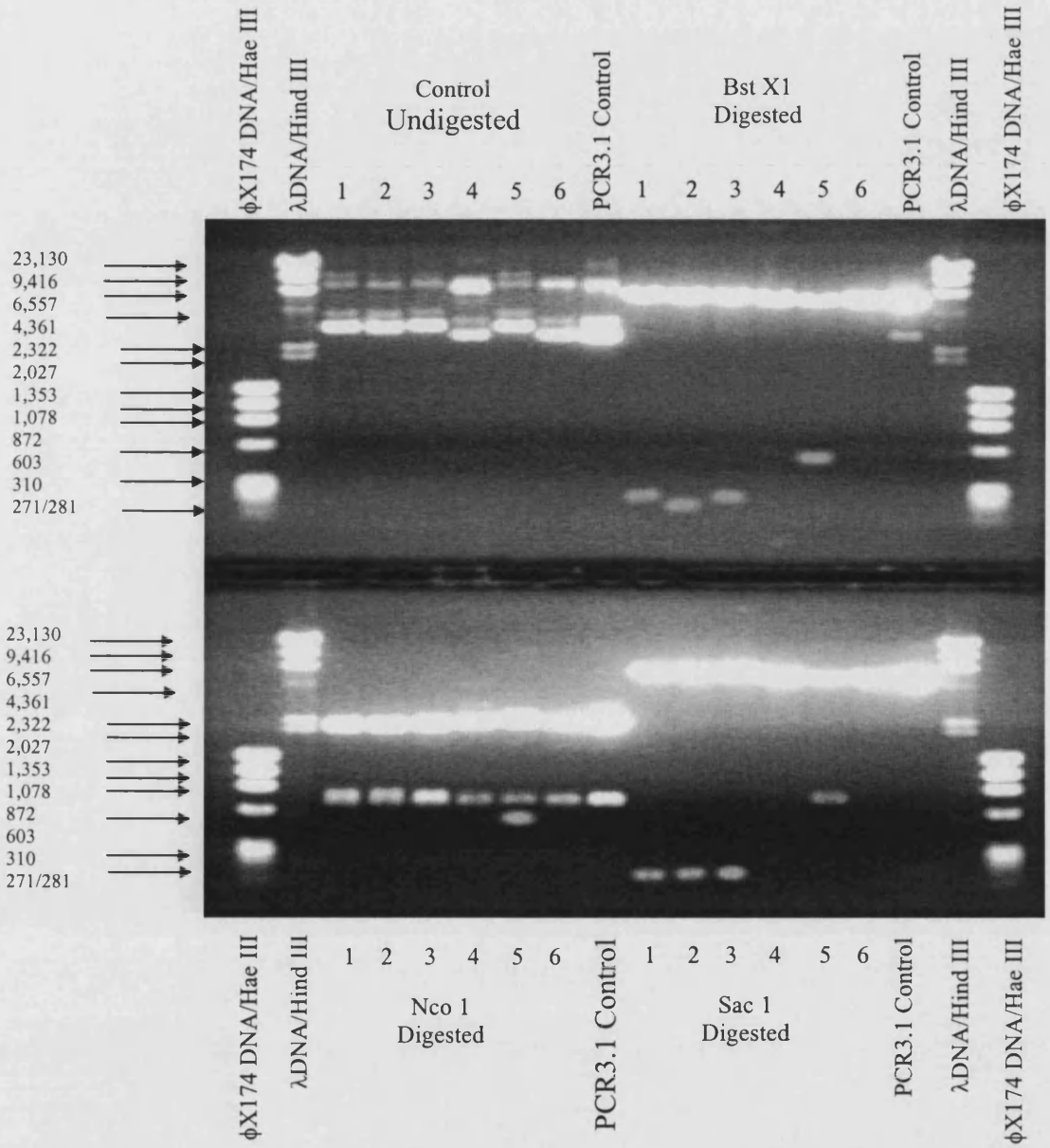


Figure 7.7: pCR3.1-CD69 Restriction Digests

Orientation of CD69 insert DNA was assessed using the restriction enzymes Nco 1, Sac 1 and Bst X1.

As can be seen in **figure 7.7** test samples one to six and control pCR3.1 circularised vector all show clear bands of coiled and supercoiled DNA in the undigested lanes. The lower bands in lanes one to three and five also appear to be retarded indicating the presence of the insert. All of the digestion reactions can be seen to have linearised the DNA tested due to the absence of the bands present between 3,500 bp and 6,500 bp in the controls. In the case of BstX1, the linearised plasmid DNA can be visualised at about 5,000 bp. Bst X1 digestion also liberated low molecular weight fragments from samples in lanes one to three indicating that the CD69 is indeed orientated in a forward direction. These data is complemented by the Nco 1 digestion, which results in bands at 697 bp and 780 bp for the samples in lanes one to three. Finally the Sac 1 digestion confirms that the plasmids in lanes one to three contain CD69 DNA in the forward orientation due to the intense bands at 125 bp and the retardation of the higher molecular weight band which is 5467 bp. Out of the three CD69 reverse samples tested only the one in lane five appears to contain insert. The insert, however can clearly be seen to be in the reverse orientation due to the digestion fragments observed with all enzymes used (i.e. 498 bp with BstX1, 459 bp with Nco 1 and 706 bp with Sac 1).

7.3.7: Transfection of CD69 into COS-7 Cells

Plasmid DNA containing the CD69 DNA was prepared from the screened colonies via the maxi-prep system described in **method 2.8.10**. Transfection via electroporation was carried out as described in **method 2.8.11** and transient expression was assessed after thirty-six hours. The electroporation conditions were varied in respect to voltage and capacitance and the cells plated out on separate petri-dishes. The FACS analysis results for transient expression can be seen in **figure 7.8**. The cells which displayed the highest degree of transient surface expression of CD69 (15%) were then selected via immunomagnetic positive retention and cultured on for several days until confluent. Ten days after transfection the magnetic bead sorted cells were stained again for CD69 and as can be seen in **figure 7.8** expressed a low level of stable CD69. These cells were selected again using magnetic beads and re-cultured until a population that was suitable for FACS sorting had developed. After twenty-four days the surface expression of CD69 can be seen to have risen to about 45%.

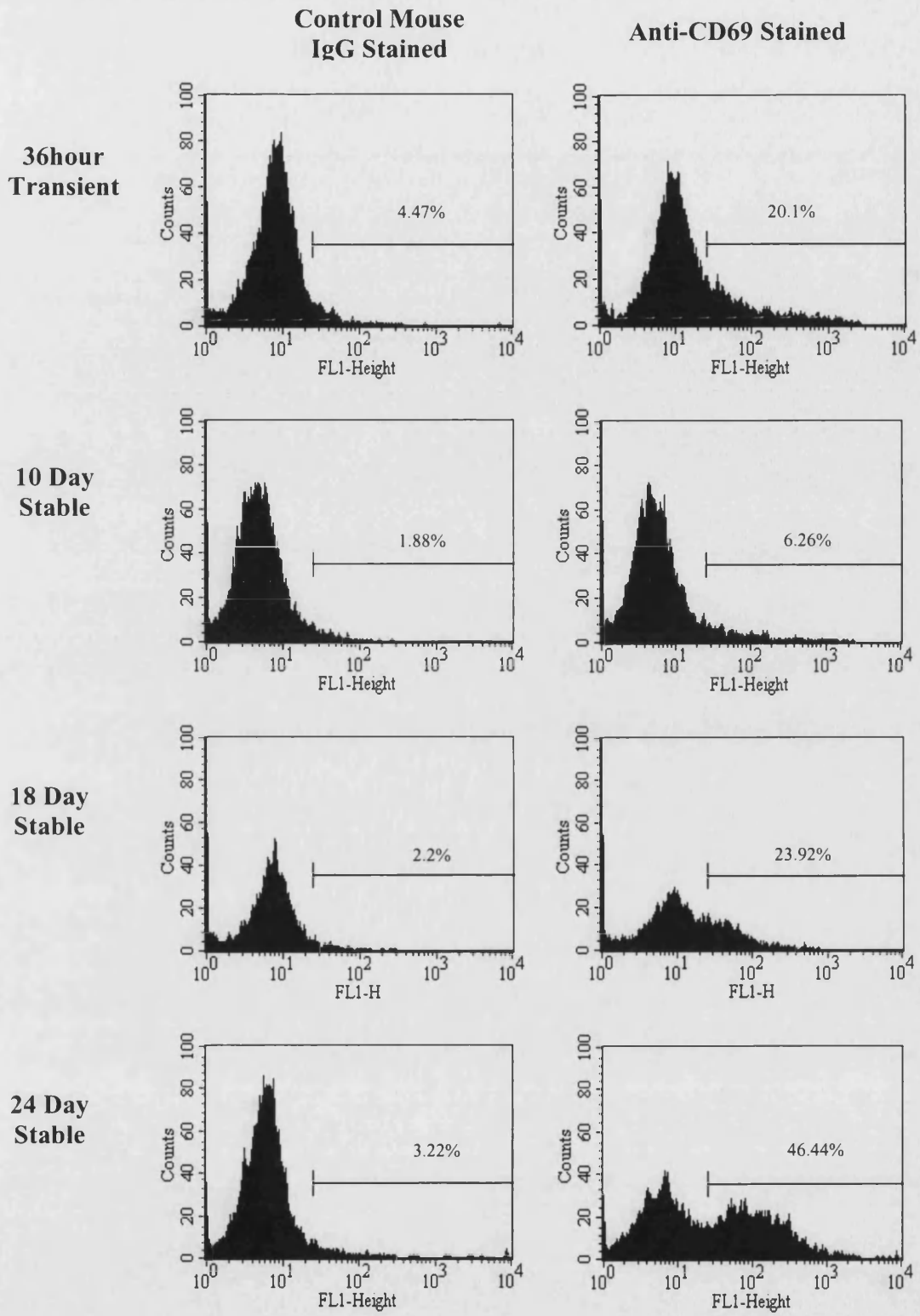


Figure 7.8: CD69 expression in transfected COS cells

7.3.8: Fluorescence Activated Selection of CD69 Positive cells

The cells that had been selected with immunomagnetic beads and determined to be 45% positive for CD69 expression were sorted using flow cytometry. After a single sort, populations of cells which were 70-80% positive for CD69 expression were recovered, as can be seen in **figure 7.9**. Various other populations were also recovered, cultured on and then frozen down for storage. The populations with the highest expression of CD69 were used for co-culture experiments to determine the effects of CD69 on the induction of D3 THP-1 IL-1β and IL-1ra.

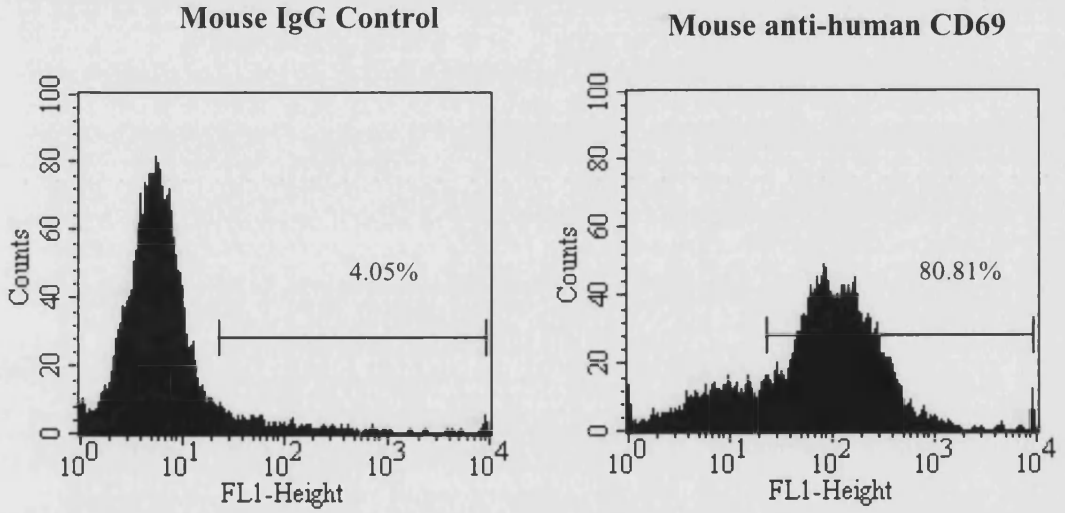


Figure 7.9: CD69 Surface Expression on COS69 line 2

7.3.9: Effect of CD69 Stimulation on D3 THP-1 Production of IL-1β and IL-1ra

D3 THP-1 cells were prepared as described in section 2.6.1 and plated out at a density of $1x10^6$/ml in 100µl volumes of RPMI-1640. To these were added 100µl of COS-69 cells that had been fixed with 0.025% glutaraldehyde and resuspended at $5x10^6$/ml in RPMI-1640. Untransfected COS-7 cells that did not express CD69 were used as controls. In some experiments D3 THP-1 cells were co-stimulated with LPS as well as membrane bound CD69.

In all experiments cells were cultured for forty-eight hours and then secreted IL-1β and IL-1ra concentrations were determined via ELISA of the supernatants (Method 2.7). Preliminary results are shown in **figure 7.10**. It must be stressed that at this point the COS-69 cells used for co-culture had varying levels of CD69 surface expression. In an attempt to repeat experiments some unsorted transfectants were used so the data displayed are merely a preliminary observation as to whether CD69 may have any effect of monocyte activation. As can be seen in the graphs, CD69 appears to synergise with LPS to induce high levels of IL-1β production from D3 THP-1 cells. COS cells transfected with CD69 were significantly better at synergising with LPS to induce IL-1β release from THP-1 cells than control COS cells ($P<0.05$). Control COS cells also had a positive effect on IL-1β production ($P<0.05$) but this could be due to the fact that COS cells express certain surface molecules that have sequence homology to human adhesion molecules. Interactions between COS cells and THP-1 cells could cause weak activation due to non-specific adhesion. The effects of CD69 on IL-1ra responses are less marked but these data needs to be improved.

7.4: SUMMARY

The main goal of this chapter was to produce a transfectant cell expressing human CD69 on its surface. Antibody binding studies using flow cytometry have clearly shown this to have been achieved. As to the role of CD69 in T cell driven monocyte cytokine production, this question will have to remain. Preliminary data suggest that CD69 is able to synergise with LPS to induce high levels of IL-1β production but more studies, with more refined transfectants, will be needed to confirm this.

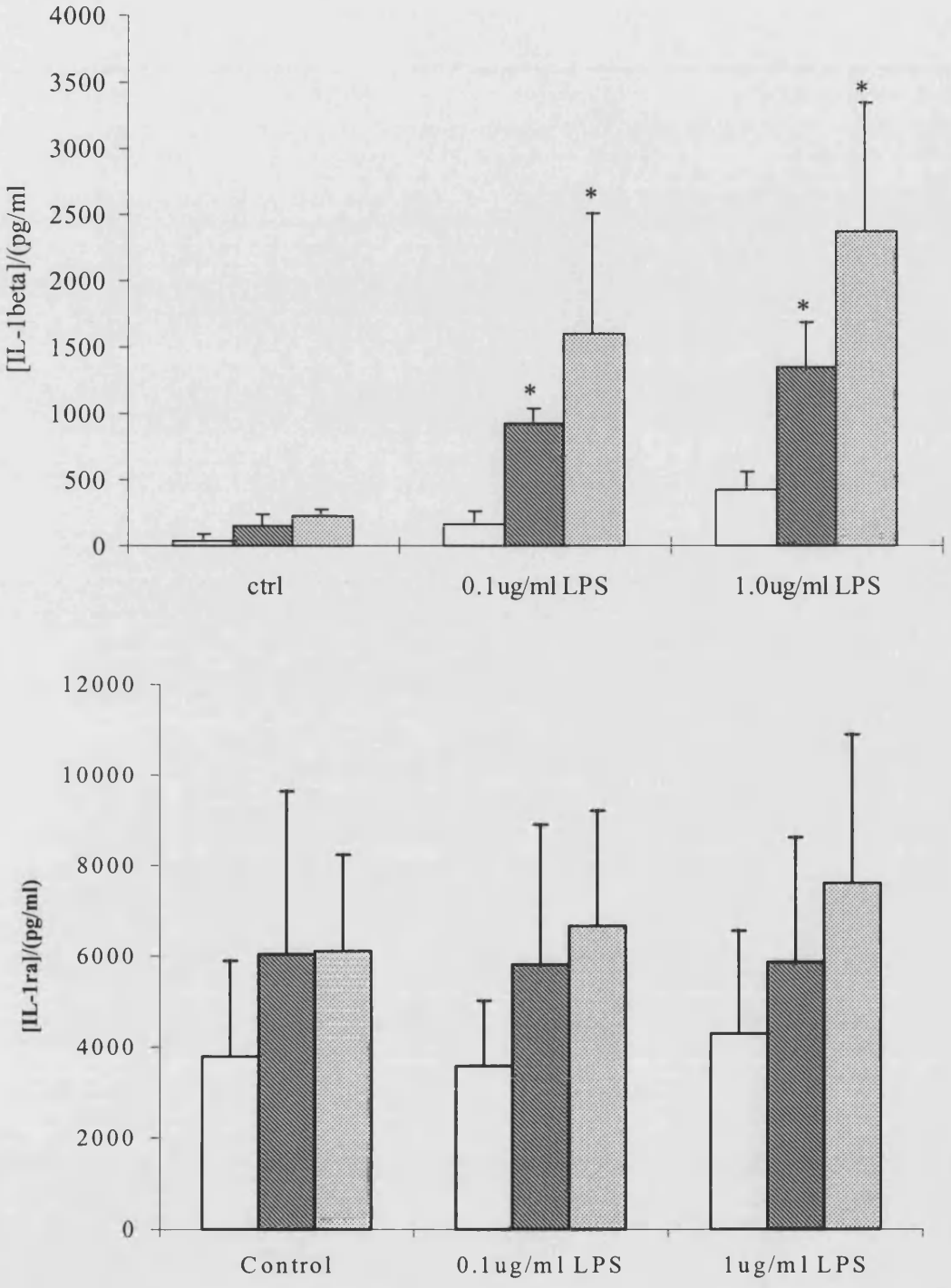


Figure 7.10: Effect of Membrane-bound CD69 Stimulation on D3 THP-1 Cytokine Production.

Panel A: IL-1β production from D3 THP-1 cells stimulated with LPS and fixed COS-69 cells for 48 hours of culture. Controls used untransfected COS-7 cells (n=3±STDEV: *=P<0.05).

Panel B: Parallel IL-1ra content of same supernatants.

CHAPTER 8

Effect of Sulphasalazine on T cell/Monocyte Co-culture

8.1: INTRODUCTION

Sulphasalazine (SPZ) is an early example of rational drug design for the treatment of rheumatoid arthritis in that the combination of a salicylate (anti-inflammatory) and a sulphonamide (antibiotic) was considered ideal for an inflammatory disease of putative bacterial aetiology. Despite fifty years of research and clinical usage, however, investigators have failed to identify a specific mode of action for SPZ in this disease (461). The predominant process leading to cartilage degradation and bone erosion is believed to be mediated largely by the prolonged generation and activity of cytokines, including TNFα and IL-1β. These proteins are produced almost exclusively by monocyte-macrophage cell types in the synovial membrane and exert potent stimulatory effects on chondrocytes and osteoclasts. The pro-inflammatory actions of TNFα and IL-1β in RA appear to be inadequately opposed by other cytokines that may be generated to limit inflammatory responses under normal conditions. Such cytokines include IL-1ra, IL-4 and IL-10. SPZ has been shown to inhibit cytokine production from human monocytes *in vitro* (518), although drug concentrations effective in these studies were significantly higher than plasma levels attained *in vivo*.

Relatively little is known about the cellular interactions or intracellular biochemistry that is associated with the imbalance of cytokine production in the chronically inflamed rheumatoid synovium. Recent research, however, has reported that co-culture of monocytes with different subpopulations of T cells promotes the production of either a broadly pro- or anti-inflammatory cytokine profile. In particular, Th1 cells can up-regulate IL-1β production by the monocytic cell line THP-1 whereas Th2 cells, which themselves produce IL-4 and IL-10, promote the generation of IL-1ra (397). Thus, a hypothesis may be proposed that one factor contributing to the chronicity of the rheumatoid lesion is an inability of the patient's T cells to drive macrophage cytokine production towards an anti-inflammatory balance. This inability could reflect either a decrease in Th2 type cytokine production or decreased contact between Th2 specific surface markers and the neighbouring macrophages. Impaired production of IL-4 (462) and IL-1ra (390) have been reported in the rheumatoid synovium.

The aim of this chapter was to explore the hypothesis that SPZ or its metabolites may influence the ability of T cells to modulate the cytokine production of

monocytic cells. As part of this work the direct effect of SPZ on T cell activation was studied as well as the effect of SPZ on control monocyte responses to IgG and LPS.

As all the studies detailed here were carried out in vitro one of the most important prerequisites of this work was to determine clinically relevant concentrations at which to use the test compounds. Consequently pharmacokinetic data compiled by Schroeder, H and Campbell, DES (1972) was utilised to determine median (and range) steady state concentrations of the parent compound and its metabolites.

Compound	**Molecular Weight**	**[Drug] in Blood with 4g/day dosing.** (median (range))/μg/ml	**[Drug] in Blood with 4g/day dosing.** (median (range))/μM
Sulphasalazine	398.39	12 (4.7-45)	15 (5-56)
Sulphapyridine	249.29	50 (18.5-46)	100 (74-185)
5-aminosalicylate	153.13	2	6

Bearing the pharmacokinetic data in mind and with advice from Pharmacia and Upjohn maximum concentrations used were 75μM SPZ, 200μM SP and 25μM ASA.

8.2: RESULTS

This chapter examines the effects of sulphasalazine (SPZ) and its metabolites sulphapyridine (SP) and 5-aminosalicylate (ASA) on monocyte cytokine production in the previously described T cell/monocyte co-culture model. Due to the complexity of this model preliminary studies examined the effects of SPZ, SP and ASA in control monocyte and T cell experiments. Monocyte responses to LPS and IgG stimulation and T cell activation studies, including surface marker expression and proliferation assays, were carried out before the effects on T cell driven monocyte cytokines were examined.

8.2.1: Effect of Sulphasalazine on LPS and IgG Driven D3 THP-1 IL-1β and IL-1ra Production

$1,25(OH)_2$-vitamin D3-differentiated THP-1 cells were used as model monocytes for the study of the effects of SPZ on the balance between IL-1β and IL-1ra production. Culture conditions were repeated as detailed before in Chapter 6 and SPZ, SP, ASA or a combination of all drugs were incubated with the D3 THP-1 cells for thirty minutes prior to stimulation. The following results are presented with IL-1β production shown in the top panels and IL-1ra production shown in the bottom panels for matched samples.

LPS induced responses are shown in **figure 8.1**. Both IL-1β and IL-1ra production are induced in a concentration dependent manner by LPS. The IL-1β and the IL-1ra responses to 1μg/ml LPS are both mildly inhibited by SPZ alone and the combination of both the parent drug and its metabolites. SP displays a mild inhibitory effect on 1μg/ml LPS induced IL-1ra ($P<0.05$) but not IL-1β and ASA has no effect at all.

Plastic bound IgG induced both IL-1β and IL-1ra production from D3 THP-1 cells in a concentration dependent manner (see **figure 8.2**). The effects of the test compounds on IgG induced IL-1β were marginal, with SPZ alone and the combination of all compounds only slightly inhibiting protein secretion. SP and ASA alone had no effect on IgG induced IL-1β production. In contrast, the large induction of IL-1ra stimulated by IgG was blocked by approximately fifty percent by SPZ. SP alone had

a weak inhibitory effect against 200μg/ml IgG induced IL-1ra and ASA had no effect against either concentration of IgG. Interestingly the combination of all the compounds had a much less effect against either concentration of IgG compared to the parent compound alone.

8.2.2: Effect of Sulphasalazine on LPS and IgG Driven Normal PBMC IL-1β and IL-1ra Production

In contrast to D3 THP-1 responses, PBMCs are much more sensitive to LPS stimulation. However, whilst LPS is a potent inducer of PBMC IL-1β production its effects on IL-1ra production are inconsistent. In D3 THP-1 basal IL-1ra release is very low, at levels of approximately 400pg/ml and LPS induces IL-1ra secretion in a dose dependent manner, reaching levels of up to 10ng/ml. In PBMC cultures, however, basal IL-1ra release often reaches levels of 6000-8000 pg/ml, perhaps due to adherence activation. In some experiments LPS has been seen to augment the release of IL-1ra from PBMCs but in others inhibition occurs.

Figure 8.3 displays the effect of SPZ and its metabolites on LPS induced IL-1β and IL-1ra production from normal PBMCs. In this experiment, LPS potently induced IL-1β production but inhibited IL-1ra release. In contrast to D3 THP-1 responses, SPZ had no effect on LPS induced IL-1β in normal PBMCs. SP, however marginally inhibited LPS induced IL-1β. As LPS did not induce IL-1ra in this experiment it is difficult to interpret the effect of SPZ under these conditions. Basal release of IL-1ra is mildly inhibited by SPZ alone and SP alone but ASA has no effect. The small inhibition of IL-1ra release induced by LPS in this model is augmented slightly by SPZ and even more so by SP. ASA has no effect on the IL-1ra responses to LPS. The combination of all compounds has no greater effect on IL-1ra responses to LPS than SP alone.

PBMC responses to IgG are more consistent than those to LPS. In all studies carried out in this thesis IgG has minimal effects on IL-1β release but strongly induces IL-1ra production. Consequently, IL-1ra production is considered a more important feature of IgG stimulation than IL-1β production. The IL-1β and IL-1ra levels produced in response to IgG are, however, displayed in parallel for comparison.

Figure 8.4 shows the very slight induction of IL-1β production seen only with 50μg/ml IgG. As the levels of IL-1β secretion are so low the slight effects seen with the test compounds are insignificant. IgG induced IL-1ra synthesis, however, did appear to be sensitive to inhibition. SPZ alone and especially SP alone inhibited IgG induced IL-1ra production but ASA had no effect. The combination of all three compounds had no greater effect than SP alone.

8.2.3: Effect of Sulphasalazine on LPS and IgG Driven IL-1β and IL-1ra Production from PBMCs Harvested from Sero-positive Rheumatoid Arthritis Patients Receiving Sulphasalazine Treatment

The effect of long-term sulphasalazine treatment was studied by measuring the responses of patient PBMCs to LPS and IgG stimulation, results being displayed in **figures 8.5** and **8.6** respectively. The possibility of cumulative effects of SPZ treatment on ex-vivo responses to SPZ and its metabolites was also considered by challenging LPS and IgG responses with the test compounds. Interestingly, although 1ng/ml LPS induced IL-1β was not affected by any of the test compounds SPZ and to a similar extent SP markedly inhibited 10ng/ml LPS induced IL-1β. Again, however, the combination of all compounds did not give an inhibition of LPS responses that exceeded that of the single compounds.

In these experiments LPS had an unusual effect on IL-1ra production. Basal IL-1ra production was comparable to levels seen in other PBMCs but was markedly elevated upon stimulation with 1ng/ml LPS. Interestingly, although 10ng/ml LPS also up-regulated IL-1ra production it was not as potent as the lower concentration of LPS. Basal IL-1ra levels were mildly inhibited by both SPZ and SP. Responses to 1ng/ml and 10ng/ml LPS induced IL-1ra, however, were slightly inhibited by SPZ but markedly inhibited by SP. The potent inhibition of SPZ against 10ng/ml LPS induced IL-1β was not paralleled with an equivalent effect against IL-1ra production. ASA demonstrated no effects against LPS induced IL-1β or IL-1ra.

The responses of PBMCs from SPZ patients to IgG were very similar to those described for normal PBMCs. As can be seen from **figure 8.6** the IL-1β induced by IgG is again very low, and although the test compounds appear to have effects the

concentrations of IL-1β that are involved are so low that no conclusions are derived from these experiments. However, the IL-1ra induced by IgG stimulation is inhibited by SPZ but more markedly so by SP. Combining the compounds had no additive effect and ASA alone had no effect on IgG induced responses.

8.2.4: Effect of Sulphasalazine on LPS and IgG Driven IL-1β and IL-1ra Production From PBMCs Harvested From Sero-positive Rheumatoid Arthritis Patients

As a control for the effects of SPZ on the responses of PBMCs from SPZ patients samples were also taken from rheumatoid patients who were not receiving second-line treatment.

As can be seen from **figure 8.7**, the effects of SPZ and its metabolites on LPS induced responses were comparable to those seen in normal PBMCs. In contrast to SPZ-patient PBMCs, control rheumatoid PBMCs showed very low sensitivity to SPZ or its metabolites. LPS induced IL-1β was not affected by any of the test compounds except a small inhibition with SP. LPS did not greatly modulate IL-1ra responses and only SP seemed to have any affect, resulting in a slight reduction in IL-1ra release.

The effects of SPZ and its metabolites on the IgG responses of rheumatoid PBMCs are shown in **figure 8.8**. In comparison to both the normal PBMCs and the SPZ-patient PBMCs the rheumatoid PBMC responses to IgG were less sensitive to SPZ or its metabolites. IgG induced IL-1β responses were comparably low and again the effects of SPZ, SP and ASA were not really significant. IgG induced IL-1ra responses, however, appeared to be less sensitive to inhibition by both SPZ and SP.

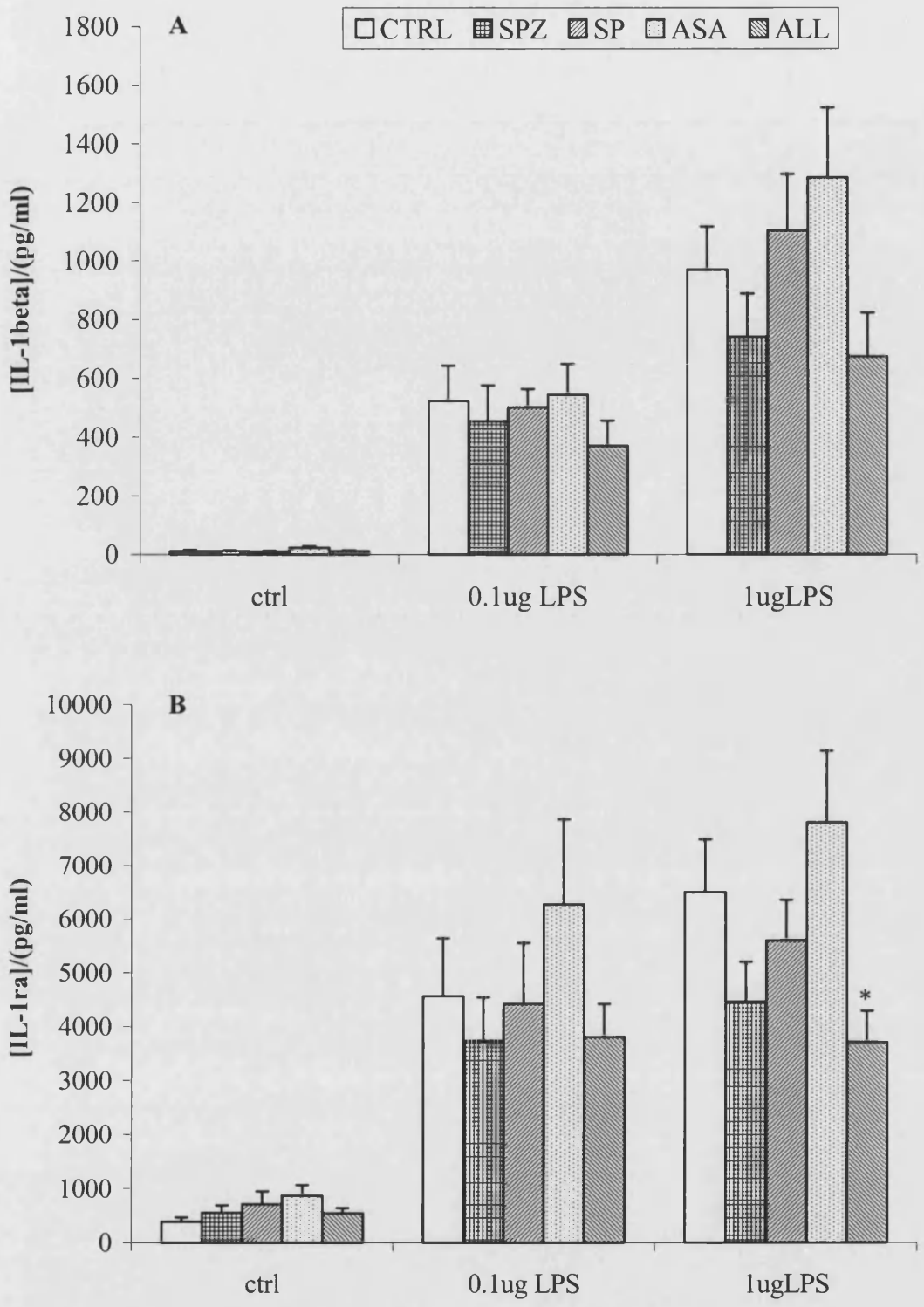


Figure 8.1: Effect of SPZ on LPS Stimulated Cytokine Release from D3-Differentiated THP-1 Cells

Panel A: Shows the IL-1β release from Differentiated THP-1 cells stimulated with 0.1 and 1μg/ml LPS for 48 hours (n=4-8 ±SEM: *=P<0.05).
Panel B: Shows parallel IL-1ra release from the same supernatants.

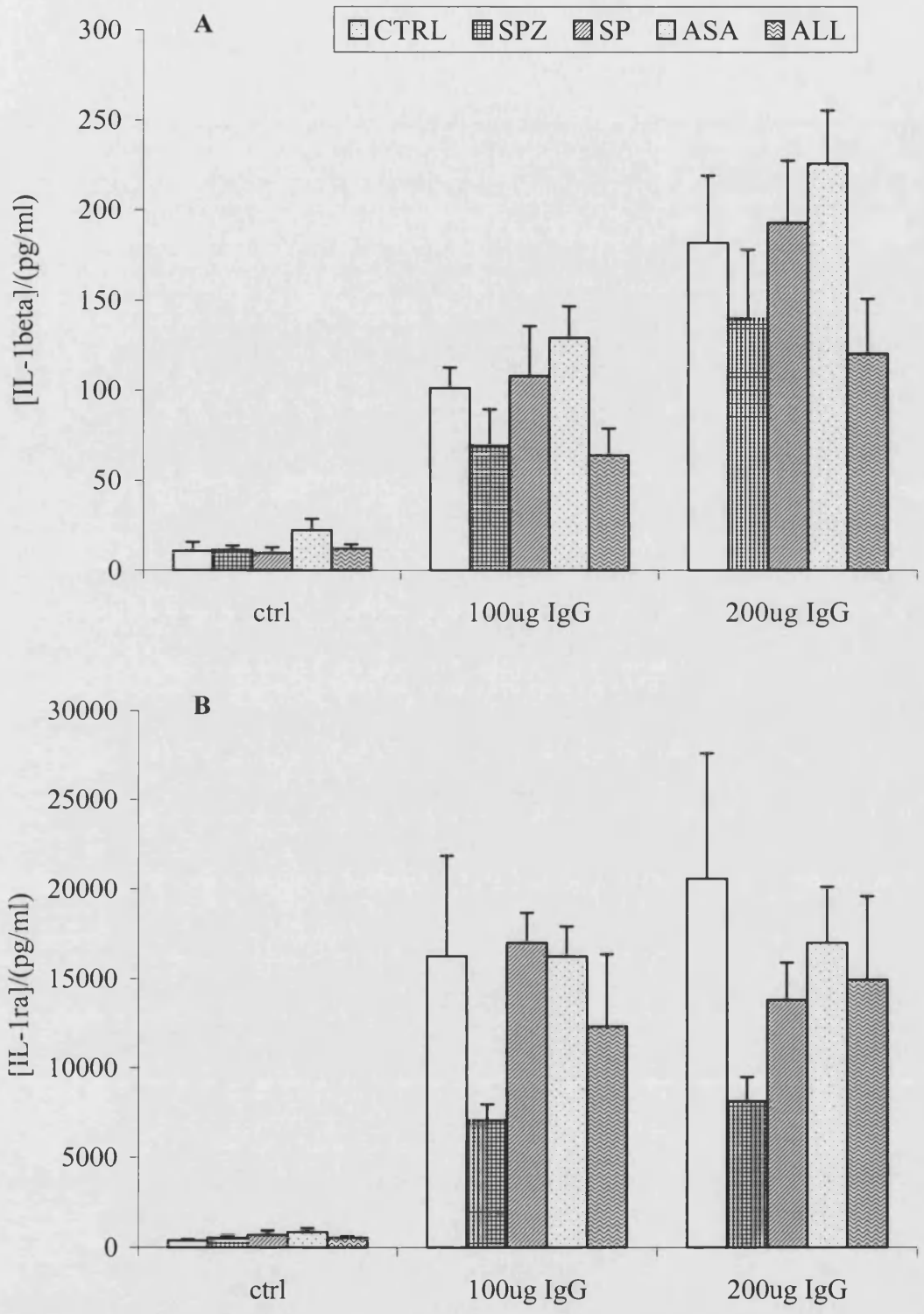


Figure 8.2: Effect of SPZ on IgG Stimulated Cytokine Release from D3-Differentiated THP-1 Cells

Panel A: Shows the IL-1β release from Differentiated THP-1 cells stimulated with plastic-bound IgG for 48 hours (n=4-8 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

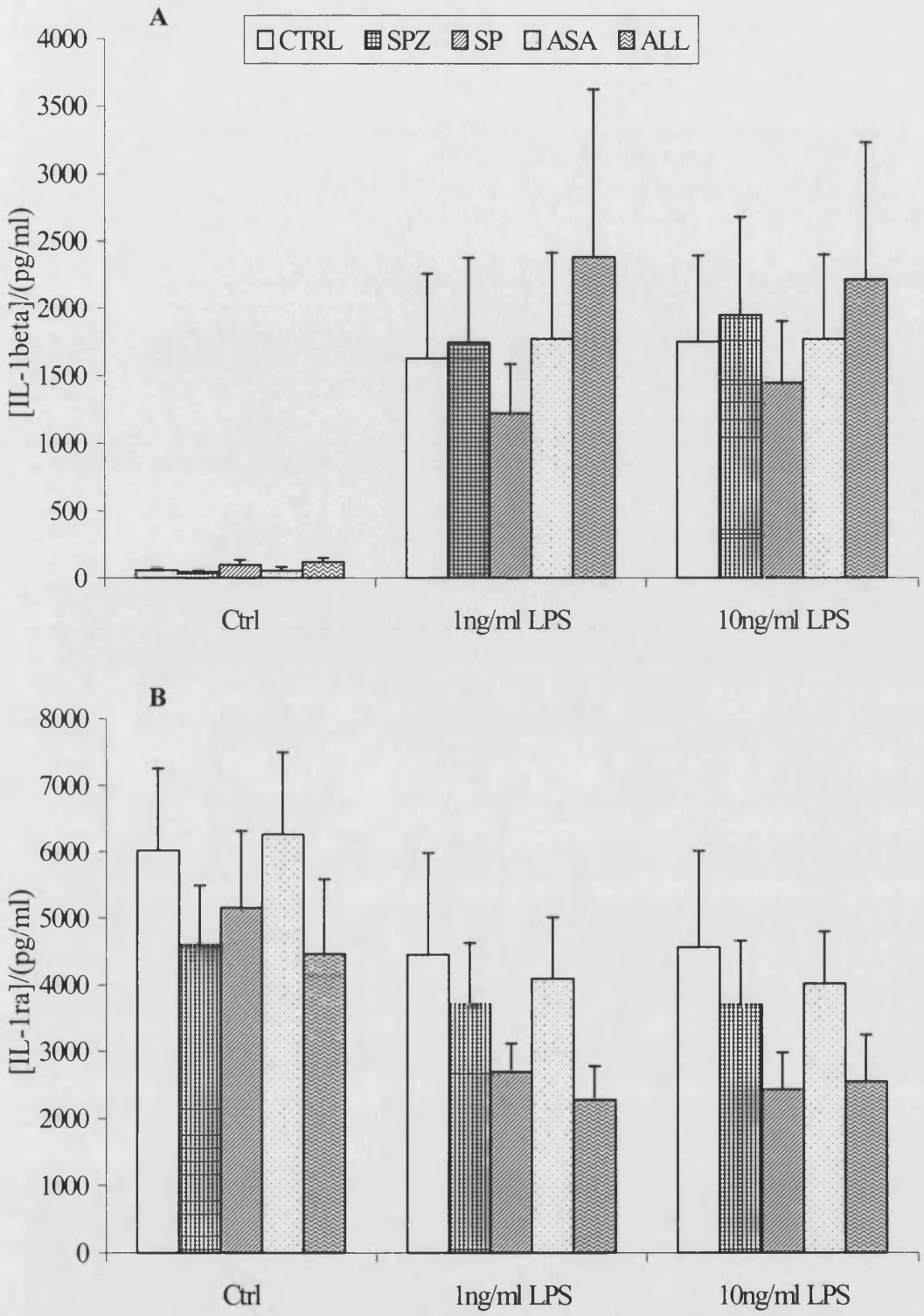


Figure 8.3: Effect of SPZ on LPS Stimulated Cytokine Release from Normal PBMCs

Panel A: Shows the IL-1β release from normal blood PBMCs stimulated with 1 and 10ng/ml LPS for 48 hours (n=6 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

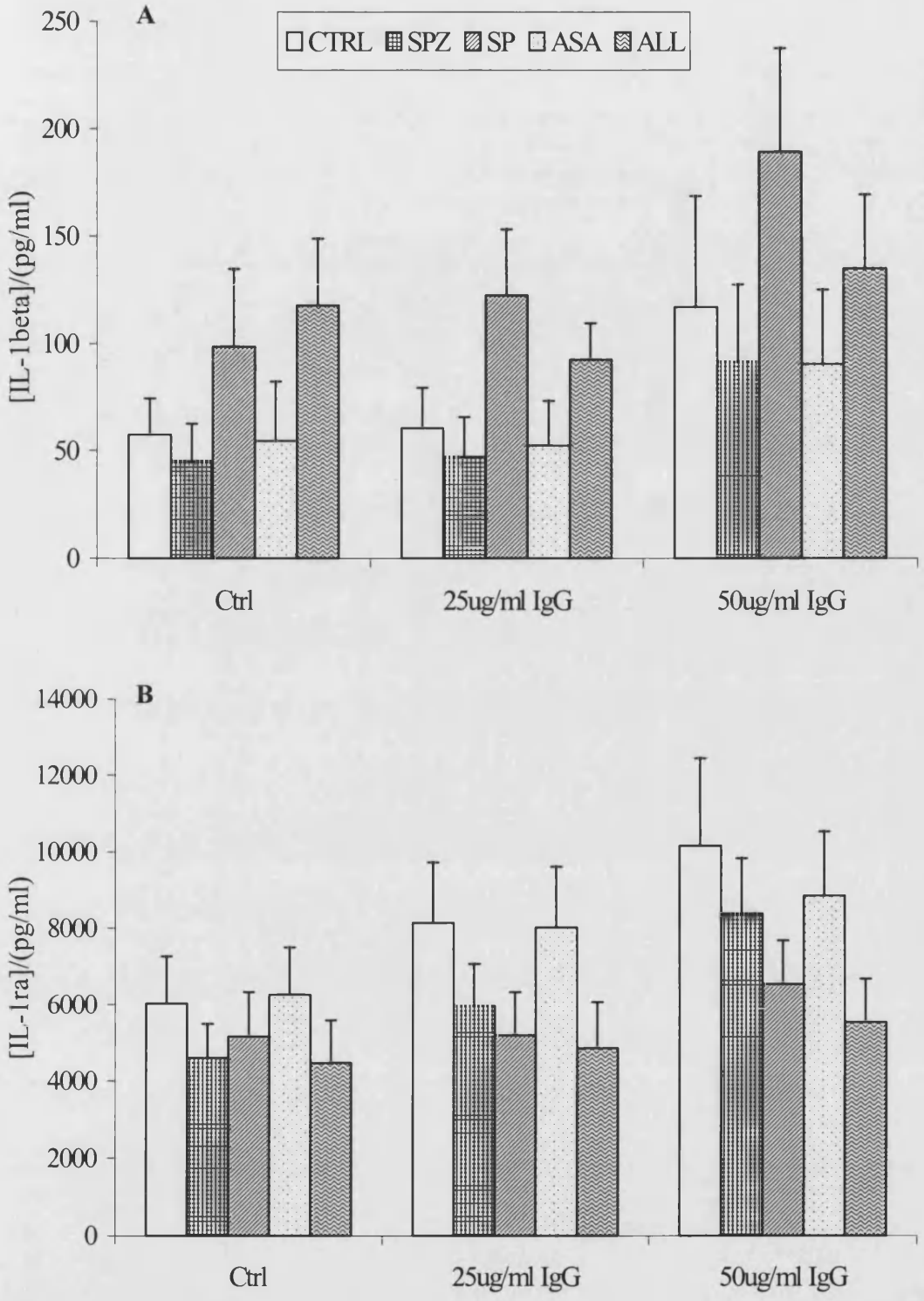


Figure 8.4: Effect of SPZ on IgG Stimulated Cytokine Release from Normal PBMCs

Panel A: Shows the IL-1β release from normal blood PBMCs stimulated with plastic-bound IgG for 48 hours (n=6 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

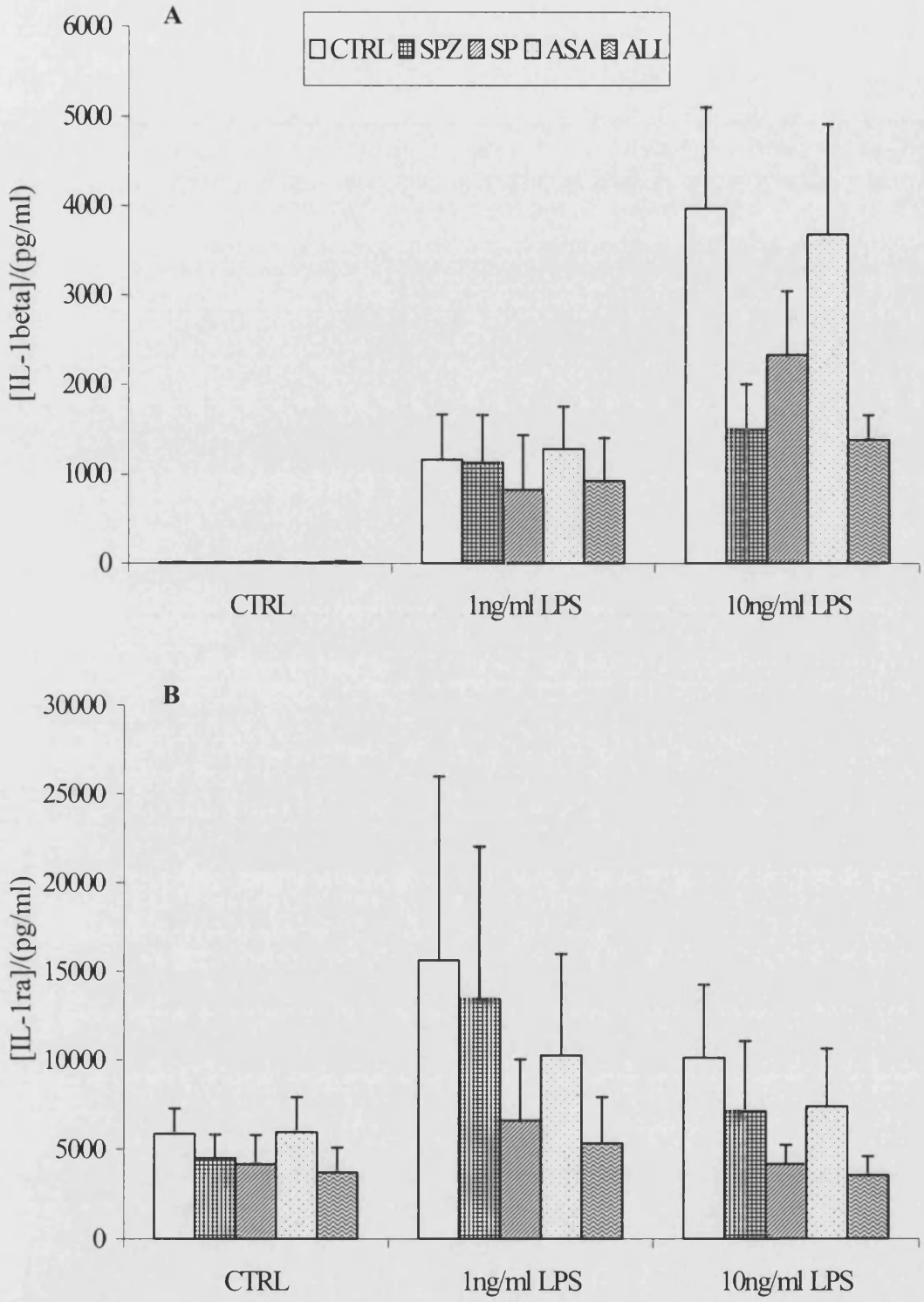


Figure 8.5: Effect of SPZ on LPS Stimulated Cytokine Release from SPZ Patient PBMCs

Panel A: Shows the IL-1β release from SPZ patient PBMCs stimulated with 1 and 10ng/ml LPS for 48 hours (n=5 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

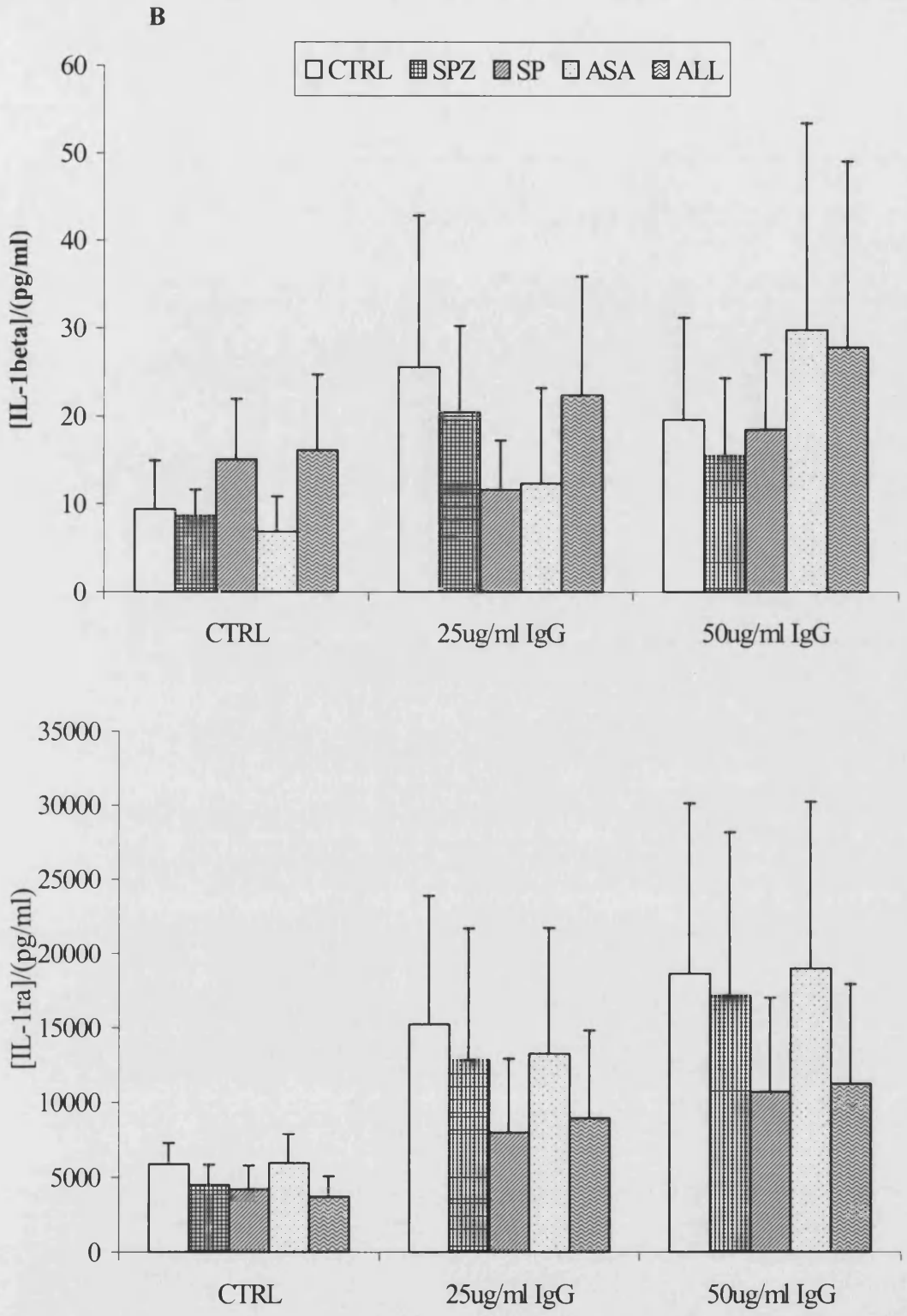


Figure 8.6: Effect of SPZ on IgG Stimulated Cytokine Release from SPZ Patient PBMCs.

Panel A: Shows the IL-1β release from SPZ patient PBMCs stimulated with plastic-bound IgG for 48 hours (n=5 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

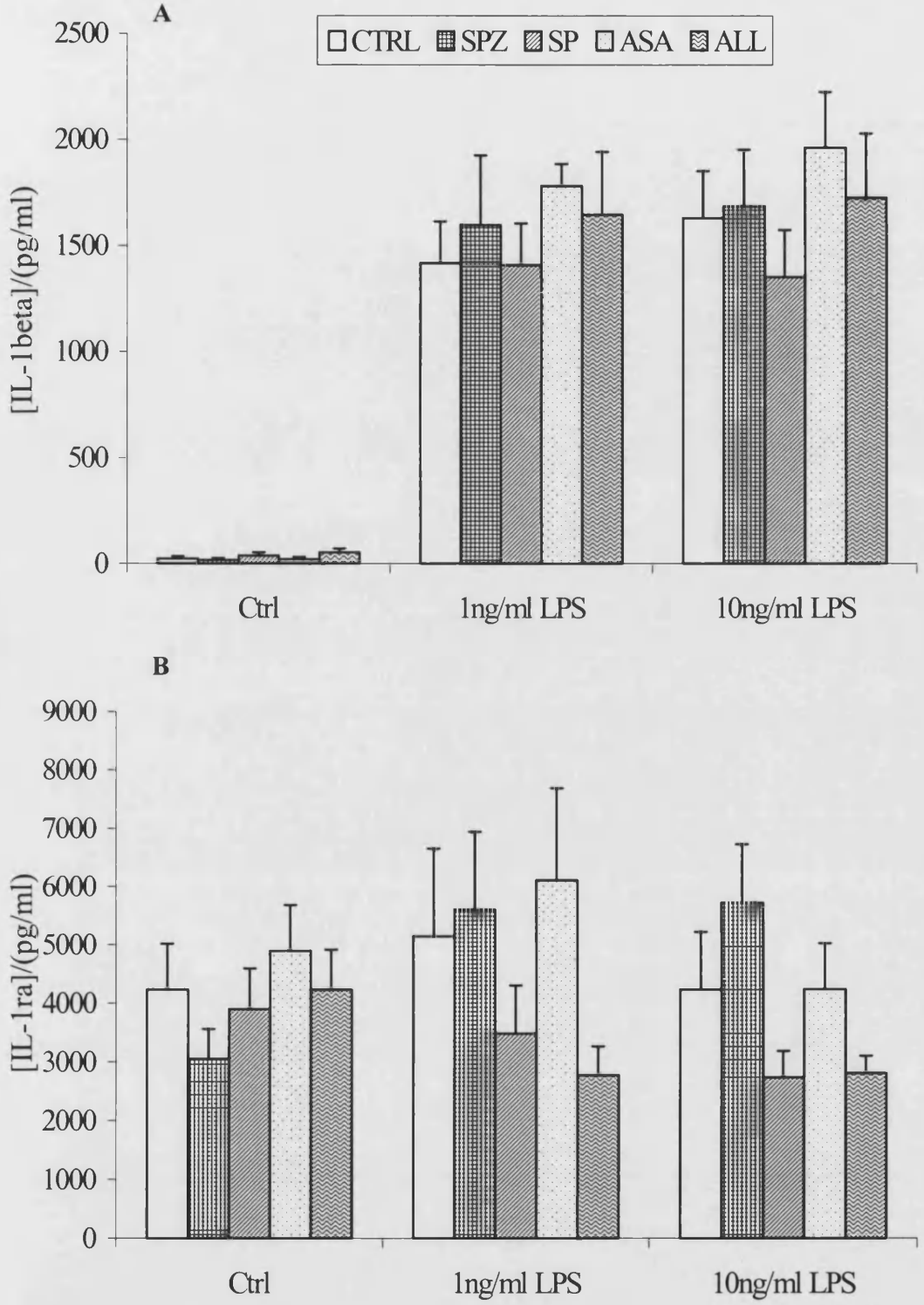


Figure 8.7: Effect of SPZ on LPS Stimulated Cytokine Release from Control SPRA Patient PBMCs.

Panel A: Shows the IL-1β release from sero-positive RA patient PBMCs stimulated with 1 and 10ng/ml LPS for 48 hours (n=7 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

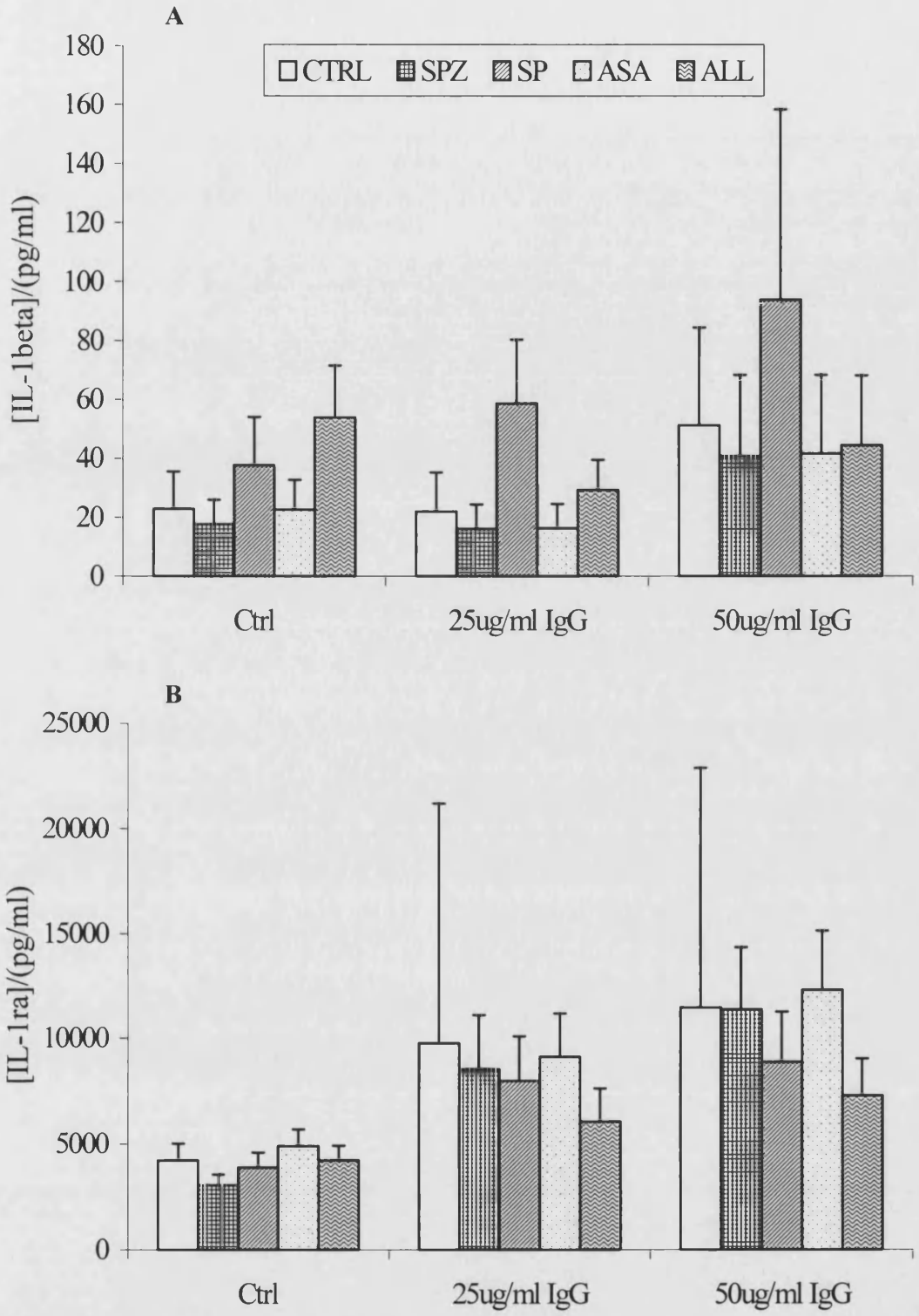


Figure 8.8: Effect of SPZ on IgG Stimulated Cytokine Release from Control SPRA Patient PBMCs.

Panel A: Shows the IL-1β release from sero-positive RA patient PBMCs stimulated with plastic-bound IgG for 48 hours (n=7 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

8.2.5: Effect of Sulphasalazine on T cell Activation

Having established control monocyte responses to LPS and IgG and studied the effect of SPZ, SP and ASA under these conditions consideration was given to direct effects of the test compounds on T cells. T cells can regulate monocyte functions via direct cell-cell contact and also through the production of various cytokines. In the co-culture model that has been developed as part of this thesis the main focus has been on T cell surface molecule derived signals and how these vary under different states of activation. Consequently, SPZ and its metabolites were assessed for their ability to modulate basic T cell proliferation responses and surface activation marker expression. For the study of the effects of SPZ on T cell driven monocyte cytokines the predominant model was to be that which utilised fixed activated T cells. Therefore the effects of SPZ on T cell derived cytokines was not considered here.

8.2.6: Effect of Sulphasalazine and its Metabolites on PDBu and Ionomycin Induced Activation Marker Expression in J16 cells

The PKC activator PDBu was used to activate J16 cells in a way that mimicked down-stream signals from TCR ligation. The calcium ionophore, ionomycin, was combined with PDBu in certain experiments to deliver a second intracellular calcium signal. In order to cover the broadest range of activation states PDBu and ionomycin concentration response curves were constructed, measuring the induction of the surface activation marker CD69. As CD69 is so rapidly induced the induction of surface expression was monitored over three time-points, two hours, four hours and twenty-two hours, to maximise the possibility of observing effects with SPZ. The effect of SPZ on PDBu and ionomycin induction of J16 CD28 expression was also monitored over a similar time course. CD28 is also regulated during T cell activation and is involved with interactions between T cells and monocytes.

J16 cells were suspended at $1x10^6$/ml in RPMI-1640 and cultured with SPZ (75μM), SP (200μM), ASA (25μM) or a combination of all three for thirty minutes prior to receiving the activation stimuli. After activation the J16 cells were removed from culture at two hour, four hour and twenty-two hour time-points and washed ready for staining with anti-CD28 and CD69 antibodies. Care was taken to halt the metabolic activity of the cells by carrying out all staining procedures at 4°C. In this way

activation induced trans-location of surface antigens to the cell membrane would be halted and the stain would reflect the surface expression at the time of cell harvest. Surface antigen expression was analysed by flow cytometry using a FITC conjugate and therefore measured as an increase in FL-1 fluorescence. **Figure 8.9A** shows a typical histogram trace for PDBu and ionomycin induced CD69 expression after twenty-two hours of culture. PDBu alone induces CD69 expression in a concentration dependent manner and synergises with ionomycin to further enhance expression of CD69. As can be seen from **figure 8.9B** neither SPZ nor its metabolites had any effect on J16 CD69 expression induced by the maximal concentrations of PDBu and ionomycin after twenty-two hours.

To simplify presentation, the rest of the data for FACS analysis of surface marker expression is displayed in table format. The degree of surface expression was monitored as an increase in Mean Fluorescence Intensity (MFI) of the whole cell population. As CD69 and CD28 induction in J16 cells involves gradual up-regulation of surface expression in the entire population rather than the development of a separate antigen positive population, the MFI is an accurate representation of the degree of surface expression. If a fluorescence level was defined as indicating positively stained cells and the percent of the population that became antigen-positive monitored instead of the total MFI, intricacies of changes in surface antigen expression on cells that have low molecule numbers would be lost. Percentage positive gating is most effective in monitoring surface expression in a heterogeneous population of cells but in this case, however, MFI measurement is the most accurate way of displaying data.

CD28 expression in J16 cells was not greatly induced by PDBu or ionomycin. Basal levels of CD28 expression were found to be unaffected by SPZ or any of its metabolites. Consequently, the data presented here in **tables 8.1, 8.2** and **8.3** only represents the effects of SPZ on activation induced CD69 expression after two, four and twenty-two hours of stimulation respectively. PDBu and ionomycin induced a time and concentration dependent increase in surface expression of CD69 but SPZ, SP and ASA had no effect.

8.2.7: Effect of Sulphasalazine and its Metabolites on PDBu and PDBu/CD80 Induced Activation Marker Expression in Normal T cells

Surface expression of CD28, CD69 and CD95 was assessed after stimulation with 5ng/ml PDBu, either alone or in combination with CD80 co-stimulation from CHO-CD80 transfectants. T cells were prepared from the blood of healthy volunteers as described in **method 2.2.6** and suspended at $1x10^6$/ml in RPMI-1640. The cells were then cultured with SPZ (75μM), SP (200μM), ASA (25μM) or a combination of all three for thirty minutes prior to receiving the activation stimuli. CHO-CD80 cells were fixed prior to use and cultured with the T cells at a ratio of 1:3.

The MFI of cells stained with anti-CD28, CD69 and CD95 is displayed in **tables 8.4, 8.5** and **8.6** respectively. Each table contains data for cells harvested at two, five and nineteen hours after stimulation, giving an indication of early and late activation marker expression. PDBu alone and in combination with CD80 had little effect on CD28 expression in the conditions used here. Basal CD28 expression climbed slightly over the nineteen-hour time-course and if anything there was a trend for CD80 to down-regulate this slightly. None of the test compounds had any effect on CD28 expression over the course of this study. Likewise, CD95 expression was not modulated by either of the activation stimuli, except for a slight elevation at the nineteen-hour time-point. Again, SPZ, SP and ASA had no effect on CD95 expression. In contrast, PDBu rapidly induced CD69 expression and the addition of CD80 augmented this induction. At the two-hour time-point SPZ, SP and ASA had no effect on either basal or activation induced CD69 expression. At the five-hour time-point, however, although statistically insignificant SPZ and SP caused a slight down-regulation of PDBu induced CD69 expression. Any effects of the test compounds were lost by nineteen hours when surface CD69 expression was at its maximum.

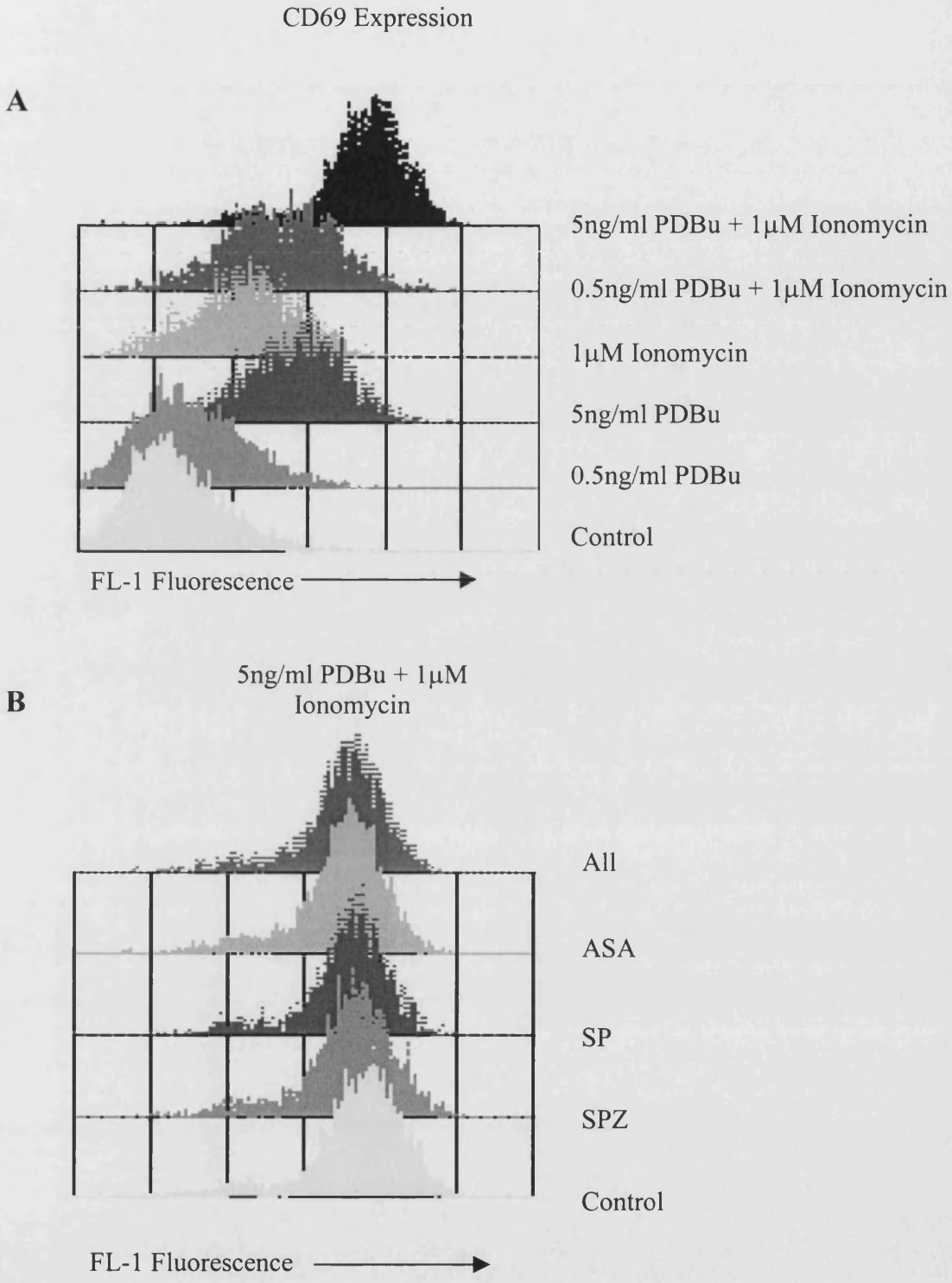


Figure 8.9: Effect of SPZ on 22 Hour Activated J16 Surface CD69 Expression.
Panel A: Effect of various stimuli on J16 CD69 expression assessed by FACS analysis.
Panel B: Effect of SPZ on 5ng/ml PDBu + 1μM Ionomycin induced CD69 expression.
Data displays the FL-1 fluorescence of entire anti-CD69 stained population and is a typical representative of three experiments.

Table 8.1: Effect of SPZ on 2 hour Activated J16 CD69 Expression (Data Represents Mean Fluorescence Intensity (MFI) of Whole Population: n=3)

Type and Duration of Stimulation	CTRL	SPZ	SP	5ASA	ALL
2 hour control	6.32	6.41	7.10	6.70	6.11
0.5ng/ml PDBu	7.13	7.43	5.96	6.39	8.46
5ng/ml PDBu	8.90	9.04	10.24	8.85	9.22
1uM Ionomycin	7.17	7.65	6.97	7.57	7.72
0.5ng/ml PDBu/I	8.74	13.59	13.14	14.30	12.88
5ng/ml PDBu/I	23.81	25.40	22.13	25.81	24.99

Table 8.2: Effect of SPZ on 4 hour Activated J16 CD69 Expression (Data Represents Mean Fluorescence Intensity (MFI) of Whole Population: n=3)

Type and Duration of Stimulation	CTRL	SPZ	SP	5ASA	ALL
4 hour control	8.37	8.54	7.93	9.08	9.18
0.5ng/ml PDBu	8.83	8.74	8.07	8.73	10.26
5ng/ml PDBu	14.25	14.20	11.65	15.54	16.69
1uM Ionomycin	10.63	10.13	8.62	10.59	11.86
0.5ng/ml PDBu/I	14.41	15.35	11.88	15.69	16.80
5ng/ml PDBu/I	27.75	36.02	30.50	30.25	32.13

Table 8.3: Effect of SPZ on 22 hour Activated J16 CD69 Expression (Data Represents Mean Fluorescence Intensity (MFI) of Whole Population: n=3)

Type and Duration of Stimulation	CTRL	SPZ	SP	5ASA	ALL
22 hour control	6.51	7.30	7.40	6.49	6.72
0.5ng/ml PDBu	8.43	9.76	8.89	8.58	7.99
5ng/ml PDBu	34.15	37.17	35.93	35.88	34.36
1uM Ionomycin	16.95	15.66	15.77	17.42	15.39
0.5ng/ml PDBu/I	29.70	28.64	25.36	28.14	25.40
5ng/ml PDBu/I	86.49	72.33	76.01	76.03	87.92

Table 8.4: Effect of SPZ on Normal T cell Surface CD28 Expression (MFI n=2)

Type and Duration of Stimulation	CTRL	SPZ	SP	ASA	ALL
2 hour ctrl	9.41	9.91	10.61	9.84	9.37
5ng/ml PDBu	11.22	10.9	12.17	11.71	10.48
5ng/ml PDBu + CD80	8.97	8.55	8.725	8.63	9.94
5 hour ctrl	9.28	9.48	10.56	9.43	10.16
5ng/ml PDBu	11.14	10.49	10.90	9.56	11.37
5ng/ml PDBu + CD80	7.98	9.01	9.23	16.6	7.73
19 hour ctrl	14.32	13.01	12.41	12.62	11.53
5ng/ml PDBu	14.47	13.6	13.23	15.76	14.46
5ng/ml PDBu + CD80	9.90	9.75	9.55	9.64	9.67

Table 8.5: Effect of SPZ on Normal T cell Surface CD69 Expression (MFI n=2)

Type and Duration of Stimulation	CTRL	SPZ	SP	ASA	ALL
2 hour ctrl	6.27	6.33	6.03	6.65	6.07
5ng/ml PDBu	17.95	18.1	18.57	19.2	17.45
5ng/ml PDBu + CD80	19.5	19.46	20.63	20.8	20.69
5 hour ctrl	7.28	6.99	7.20	6.96	7.16
5ng/ml PDBu	33.55	26.08	24.12	28.55	24.33
5ng/ml PDBu + CD80	31.77	31.54	25.14	32.88	30.62
19 hour ctrl	20.65	14.17	12.4	12.37	13.2
5ng/ml PDBu	39.16	36.54	40.89	45.47	38.14
5ng/ml PDBu + CD80	45.49	37.32	36.72	39.18	36.78

Table 8.6: Effect of SPZ on Normal T cell Surface CD95 Expression (MFI n=2)

Type and Duration of Stimulation	CTRL	SPZ	SP	ASA	ALL
2 hour ctrl	10.32	9.31	9.85	9.05	9.08
5ng/ml PDBu	10.50	10.00	8.13	8.85	8.65
5ng/ml PDBu + CD80	9.915	10.05	10.65	11.11	9.35
5 hour ctrl	11.95	12.61	10.67	11.79	10.91
5ng/ml PDBu	10.17	9.725	9.30	9.97	10.35
5ng/ml PDBu + CD80	10.71	10.97	10.57	10.45	11.45
19 hour ctrl	15.79	15.66	15.08	14.73	15.36
5ng/ml PDBu	16.96	16.14	17.11	17.60	16.27
5ng/ml PDBu + CD80	18.53	16.82	15.54	17.51	16.21

8.2.8: Effect of Sulphasalazine and its Metabolites on T cell Proliferation

As can be seen in **figure 6.9B** J16 cells are a very rapidly growing cell line and consequently were not suited for studies on proliferation induction. The effects of SPZ and its metabolites on T cell proliferation were, therefore, only studied in normal T cells. T cell proliferation assays were carried out as described previously in **section 6.2.4** and **method 2.2.8.** Control stimulations were characterised using PDBu and aggregated anti-CD3 antibody either alone or in combination with CHO-CD80. Cells were stimulated for seventy-two hours and ^{3}H-thymidine incorporation was monitored over the last eighteen hours. Typical control proliferation responses are shown in **figure 8.10**, displaying synergistic induction of proliferation with CD3/CD80 and PDBu/CD80.

The T cells were cultured with SPZ (75μM), SP (200μM), ASA (25μM) or a combination of all three for thirty minutes prior to receiving the activation stimuli. CHO-CD80 cells were fixed prior to use and cultured with the T cells at a ratio of 1:3.

As well as activation induced proliferation, basal T cell proliferation was also monitored in order to check for cytotoxic drug effects. As can be seen in **figure 8.11A**, none of the compounds alone had any effect on basal T cell proliferation. The combination of all three compounds, however, mildly suppressed basal proliferation but with the low number of experiments carried out this proved to be insignificant. A similar trend was observed with CD3/CD80 stimulation (**figure 8.11B**) with only the combination of all three compounds having any effect. In contrast, PDBu/CD80 induced proliferation was most resistant to modulation by any of the test compounds with not even the combination of drugs being effective (**figure 8.12A**).

Interestingly, it appeared that any effects of the test compounds on T cell proliferation were seen in the combination group only. Both basal proliferation and the CD3/CD80 stimulation were mildly inhibited but the more aggressive pharmacological induction of proliferation using PDBu was unaffected. It is possible that the more physiological type proliferation induced by CD3 stimulation was more sensitive to inhibition by the combination of SPZ and its metabolites. Complementary data was also observed in three experiments when it was noticed that CD80 stimulation alone induced a mild proliferation. In these CD80 responders

SPZ, SP and even ASA had a marked effect on proliferation, inhibiting ^{3}H thymidine incorporation by about 50% (**figure 8.12B**).

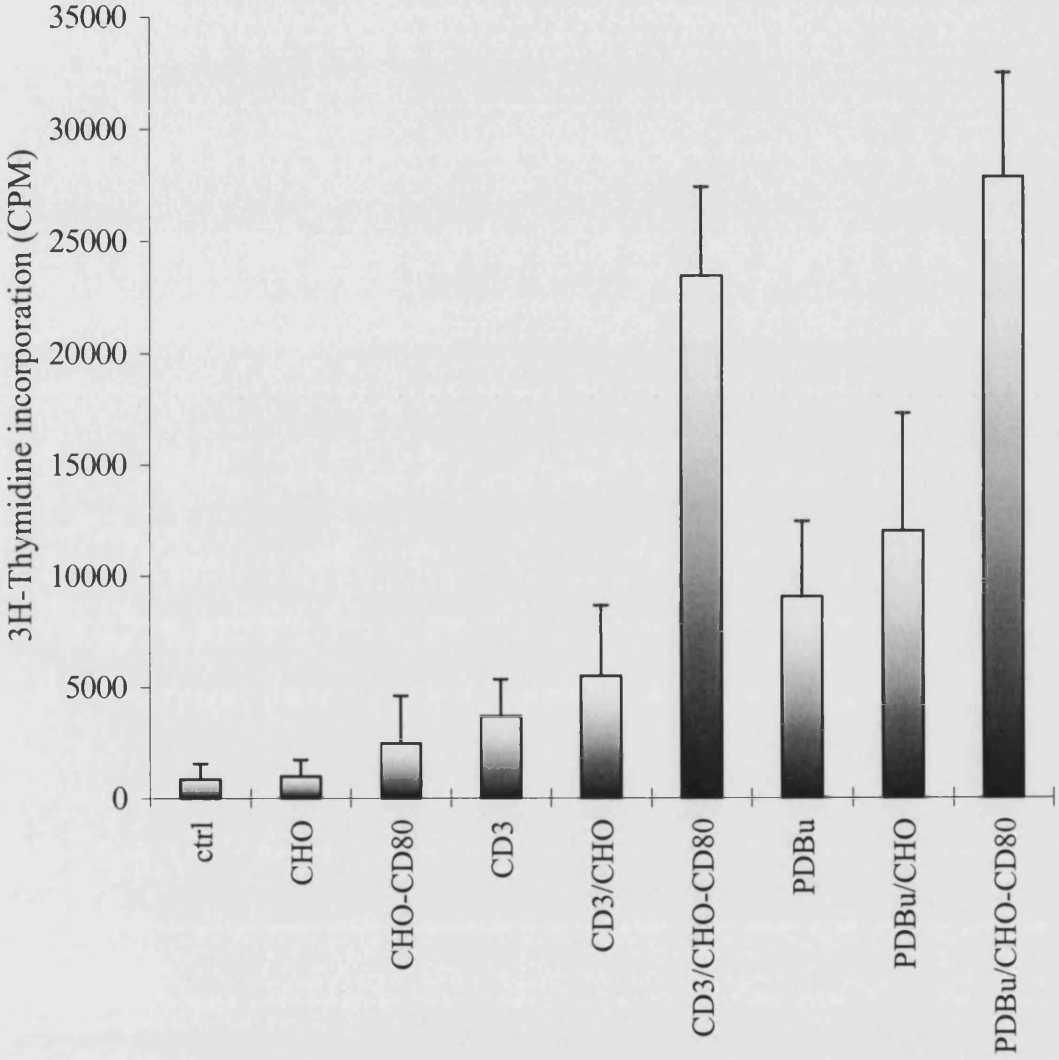


Figure 8.10: Control Proliferation Responses of Normal T cells

Normal T cells were stimulated with 5ng/ml PDBu or 1μg/ml anti-CD3 with or without CHO-CD80. Cells were cultured for seventy-two hours and the incorporation of ^{3}H thymidine was measured over the last eighteen hours (Data represents the mean of four experiments ± STDEV).

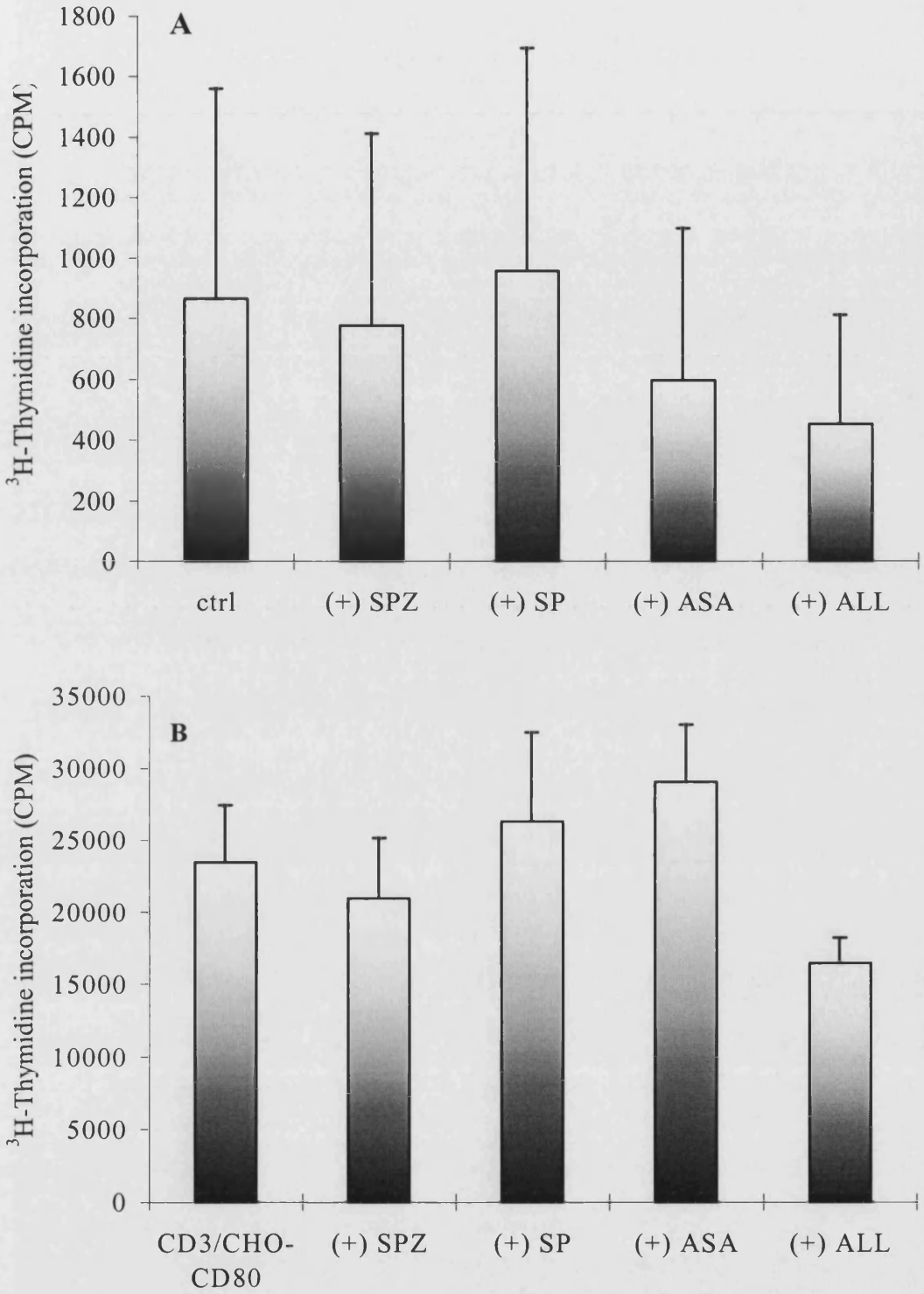


Figure 8.11: Effect of SPZ, SP and ASA on Normal T cell Proliferation
Panel A: Effect of SPZ on basal proliferation of resting T cells.
Panel B: Effect of SPZ on CD3/CD80 induced T cell proliferation. Normal T cells were stimulated with 1μg/ml anti-CD3 and CHO-CD80. Cells were cultured for seventy-two hours and the incorporation of ^{3}H thymidine was measured over the last eighteen hours (Data represents the mean of four experiments ± STDEV).

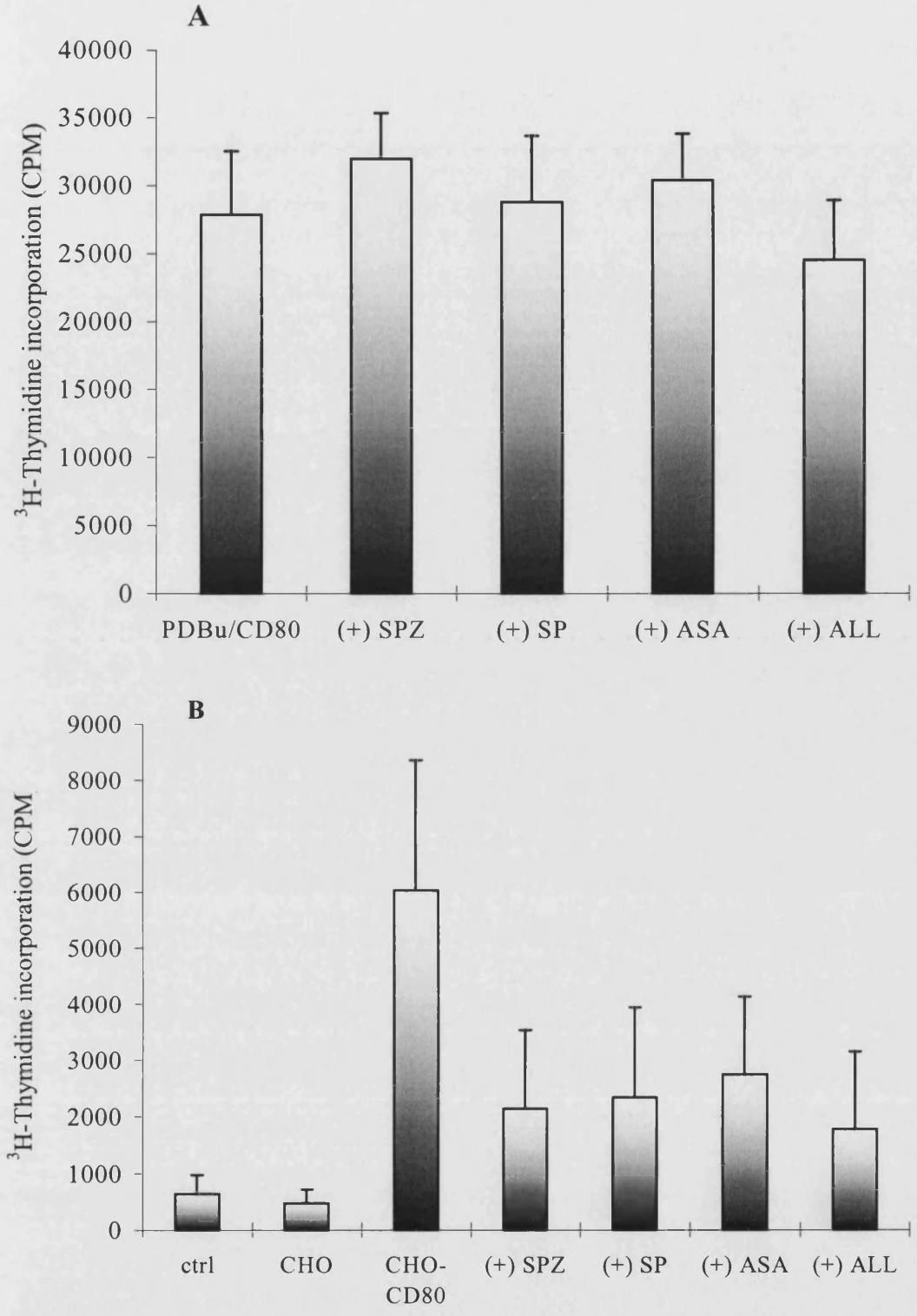


Figure 8.12: Effect of SPZ, SP and ASA on Normal T cell Proliferation
Panel A: Normal T cells were stimulated with 5ng/ml PDBu and CHO-CD80 (Data represents the mean of four experiments ± STDEV).
Panel B: Normal T cells were stimulated with CHO-CD80 alone (Data represents the mean of three experiments ± STDEV).
Cells were cultured for seventy-two hours and the incorporation of ^{3}H thymidine was measured over the last eighteen hours

8.2.9: Effect of Sulphasalazine on T cell Driven Monocyte IL-1β and IL-1ra Production

Fixed activated J16 cells were used to stimulate cytokine production in D3 THP-1 cells, normal PBMCs, SPZ-patient PBMCs and control rheumatoid PBMCs. Fixation of the J16 cells ruled out any contribution of soluble factors and thus the "T cell" stimulus was defined as a membrane interaction only. The test compounds, SPZ, SP and ASA were pre-incubated with the monocytes for thirty minutes prior to stimulation with the J16 cells.

Figure 8.13 shows J16 induction of IL-1β and IL-1ra production in D3 THP-1 cells. As can be seen from both panels J16 cells induced IL-1β and IL-1ra in an activation dependent manner, with resting J16 cells having little effect on cytokine production. However, neither SPZ nor its metabolites SP and ASA had any effect upon J16 induced IL-1β or IL-1ra production from D3 THP-1 cells.

With PBMCs, fixed activated J16 stimulation resulted in a small increase in IL-1β production and a more pronounced increase in IL-1ra production. The cytokine production induced in normal PBMCs is shown in **figure 8.14**. Similarly to IgG stimulation the IL-1β response of normal PBMCs when co-cultured with activated J16 cells reflects a subtle elevation in cytokine production. There is a three to five-fold increase in IL-1β production above that seen in controls, but this represents an increase in concentration of only 100 to 150pg/ml. As a result, the interpretation of the effect of SPZ and its metabolites on J16 induced IL-1β production is difficult. Naturally, as this drug has been seen to be efficacious in the treatment of RA for some fifty years it would be strange to expect to see a pro-inflammatory cytokine profile induced in monocyte cultures. However, with J16 stimulated normal PBMCs that is what is observed. Both SPZ and more markedly SP slightly augment J16 induced IL-1β production and inhibit IL-1ra production. Although these effects are statistically insignificant there is a clear trend and the question that is left is whether this degree of elevation of IL-1β production is truly biologically significant. If SPZ is having such an effect on IL-1β production, albeit a modest one, could it perhaps be due to experimental conditions or toxicity?

In contrast to normal PBMCs, PBMCs taken from SPZ patients display different responses to SPZ in vitro. As can be seen in **figure 8.15** activated fixed J16 cells

stimulated both IL-1β and IL-1ra production. However, a very small but equally non-significant inhibition, rather than an augmentation of IL-1β production was seen with SPZ and SP treatment. In contrast, like in normal PBMCs SPZ and SP mildly inhibited J16 induced IL-1ra production.

Control rheumatoid PBMCs gave another variable response to SPZ treatment when challenged with activated J16 cells (see **figure 8.16**). SP and to a similar extent ASA slightly augmented J16 induced IL-1β but none of the compounds had any effect on J16 induced IL-1ra production.

8.2.10: General Balance Between IL-1β and IL-1ra Production Observed in the Different Patient Groups

Out of all the observations made on the effects of sulphasalazine in the co-culture models described here perhaps the most interesting is the difference between the basal responses of the different patient groups. As can be seen in **figure 8.17**, SPRA patients who have received SPZ treatment display an elevated IL-1ra response and depressed IL-1β response to all of the stimuli studied. At present with just an experimental repeat of five for the SPZ patients these data are not statistically significant but the trend is clear.

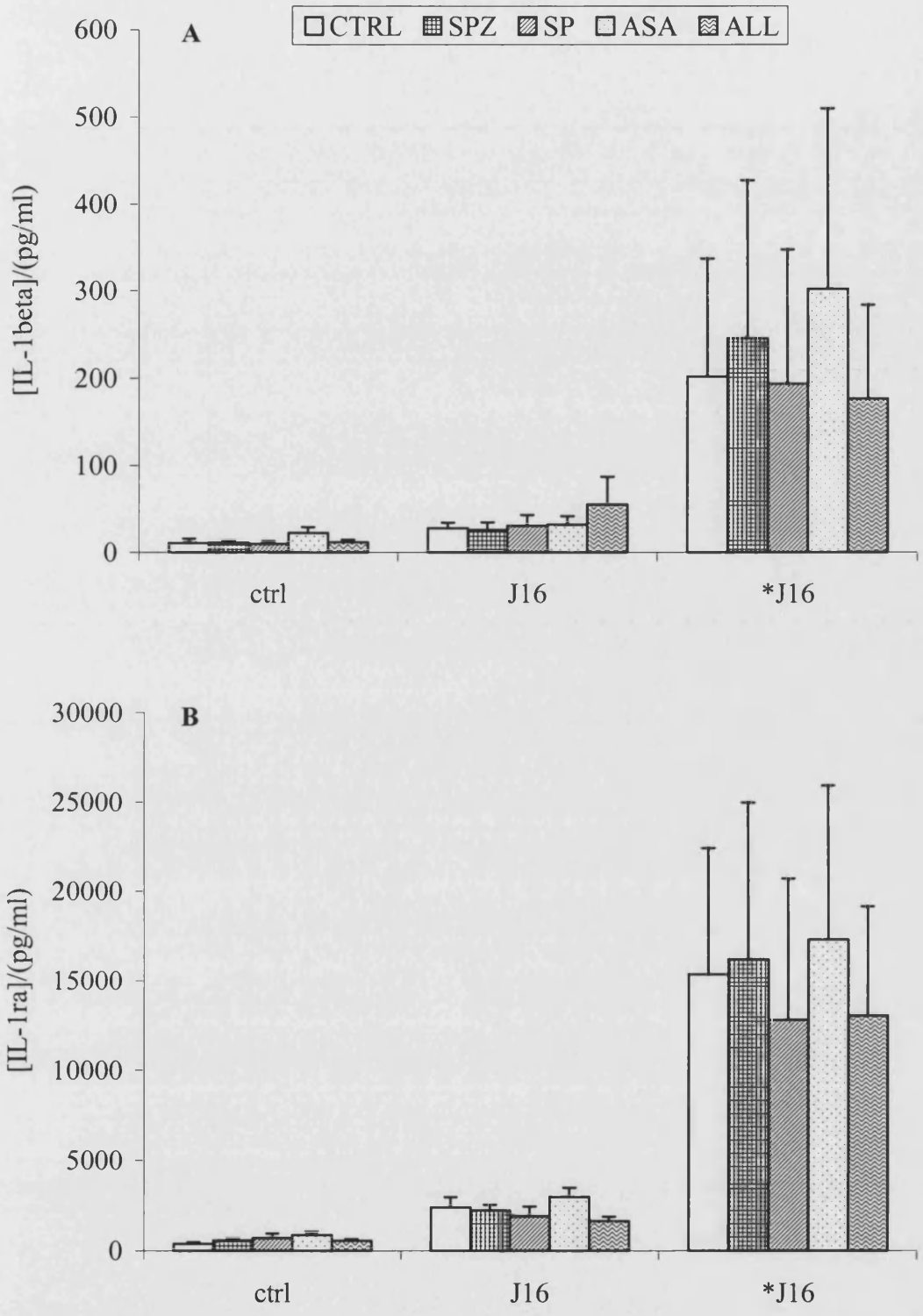


Figure 8.13: Effect of SPZ on J16 Stimulated Cytokine Release from D3-Differentiated THP-1 cells.

Panel A: Shows IL-1β release from D3 THP-1 cells cultured for 48 hours with fixed resting or activated (*) J16s(n=4-8 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

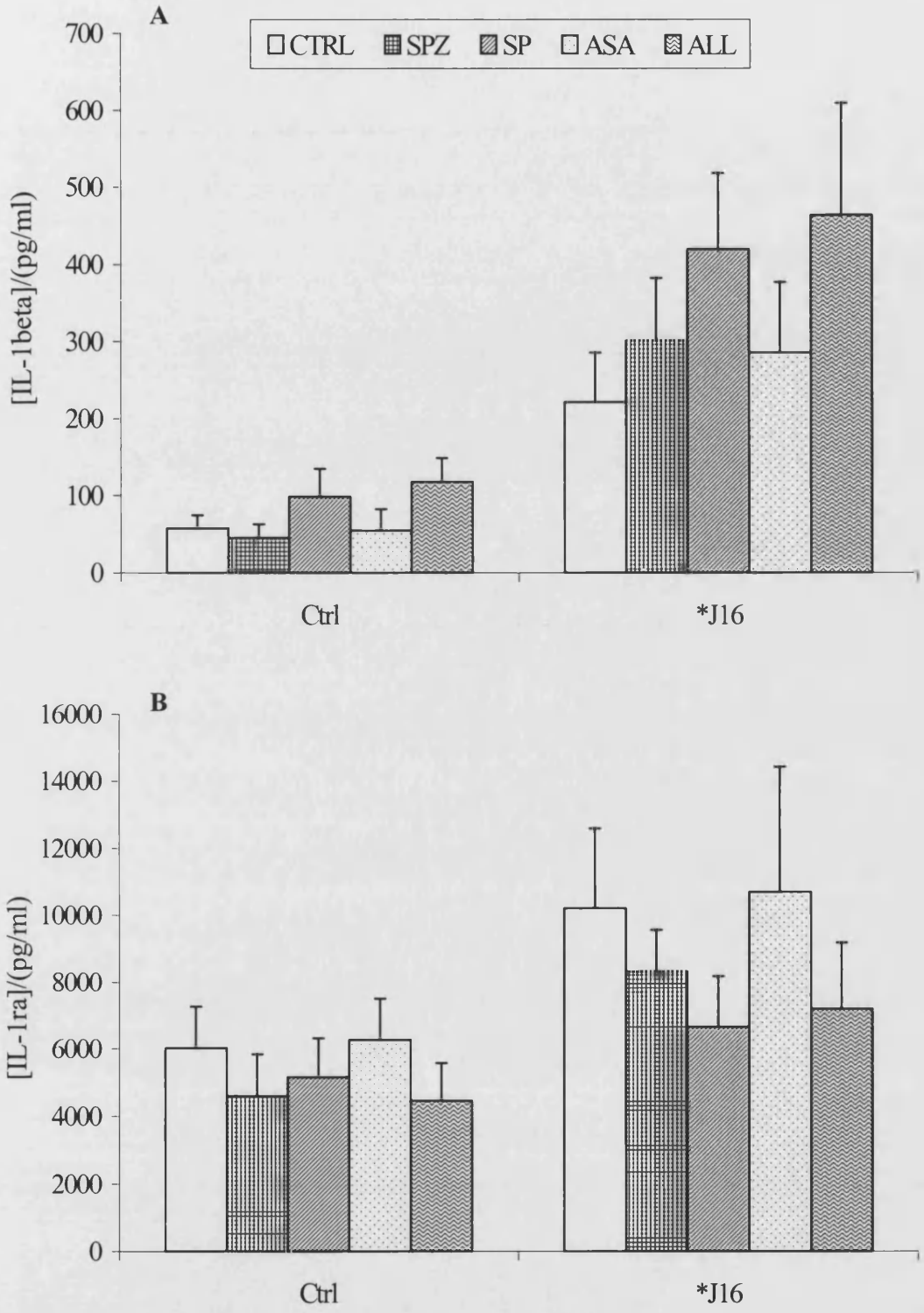


Figure 8.14: Effect of SPZ on J16 Stimulated Cytokine Release from Normal PBMCs.

Panel A: Shows IL-1β release from normal PBMCs cultured for 48 hours with fixed activated (*J16s) (n=6 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

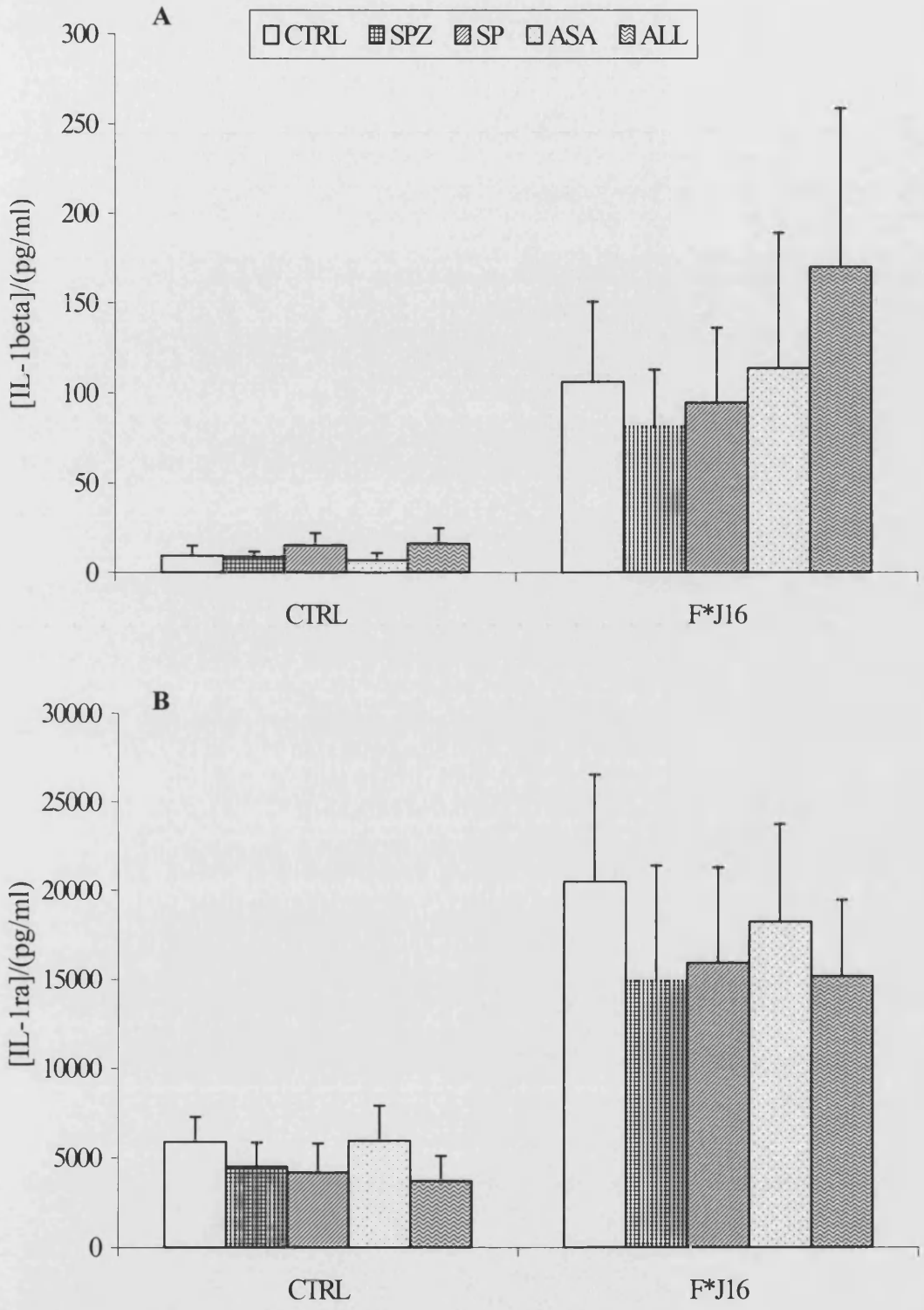


Figure 8.15: Effect of SPZ on J16 Stimulated Cytokine Release from PBMCs from SPZ Patients

Panel A: Shows IL-1β release from SPZ patient PBMCs cultured for 48 hours with fixed activated (*J16s) (n=5 ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

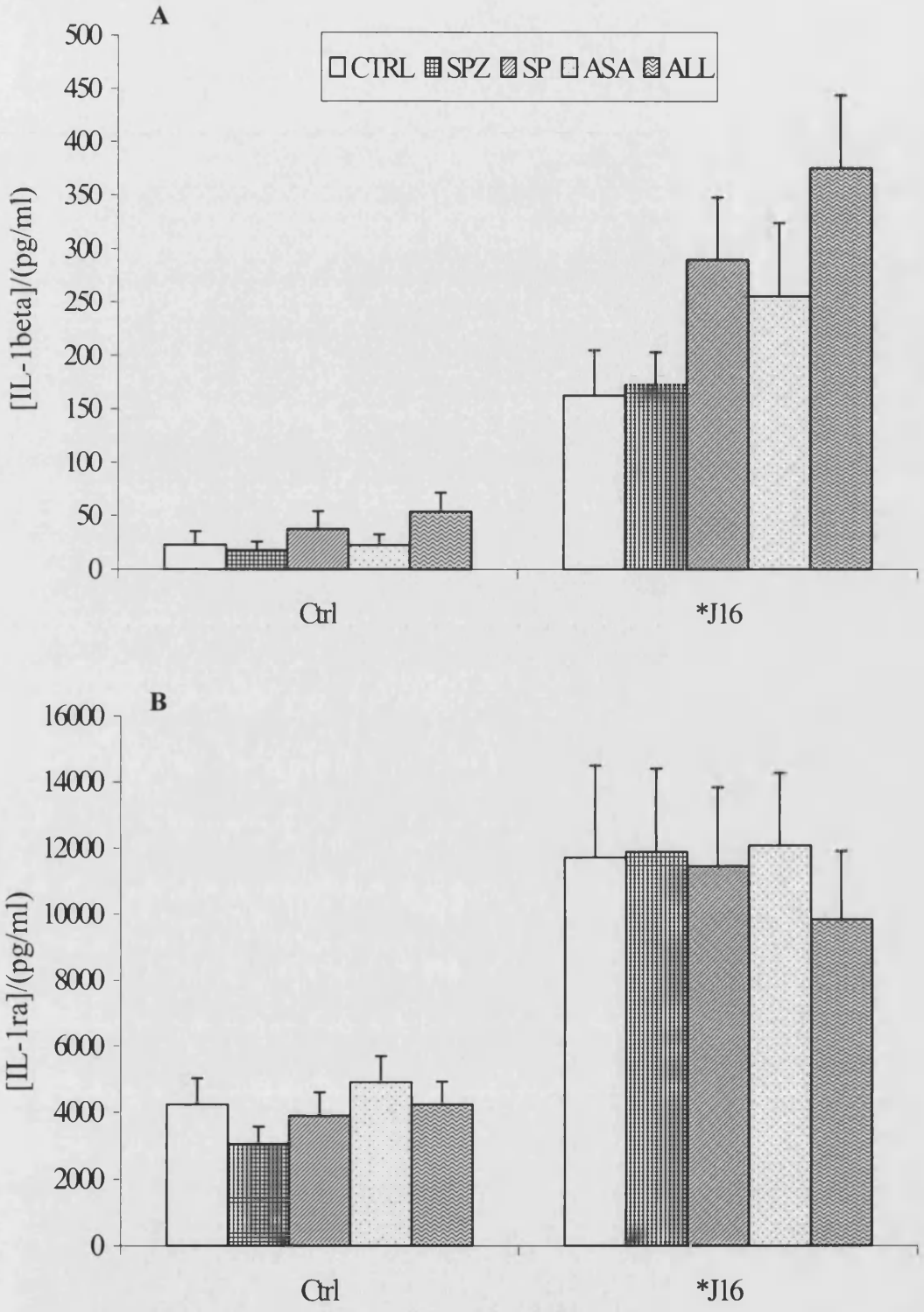


Figure 8.16: Effect of SPZ on J16 Stimulated Cytokine Release from SPRA Disease Control PBMCs

Panel A: Shows IL-1β release from PBMCs cultured for 48 hours with fixed activated (*J16s). PBMCs were purified from sero-positive RA patients attending clinics at the RNHRD who were not taking second-line treatments (n=7, ±SEM).
Panel B: Shows parallel IL-1ra release from the same supernatants.

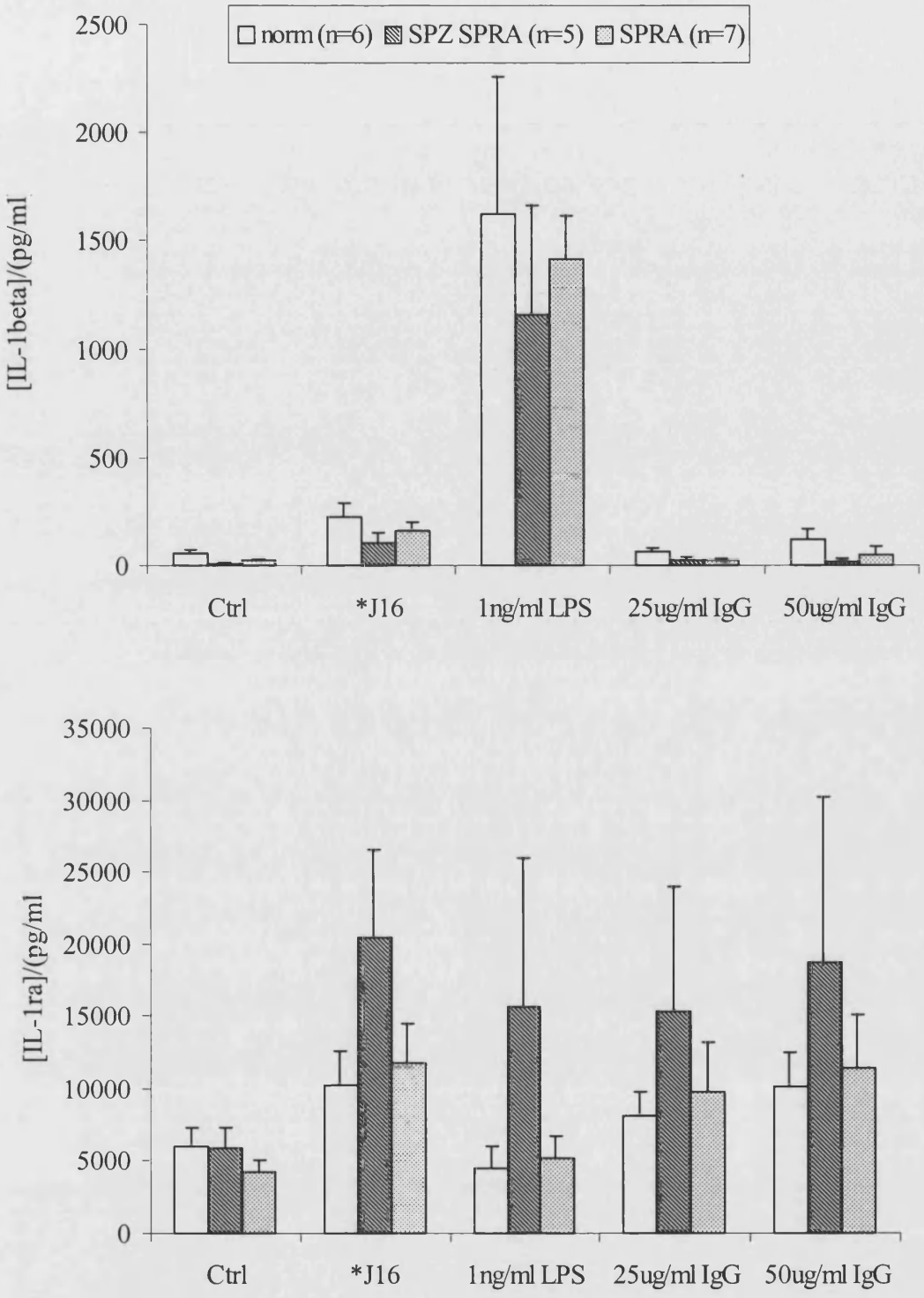


Figure 8.17: Comparison of Control IL-1β and IL-1ra Responses of SPZ Patient PBMCs, Control SPRA PBMCs and Normal PBMCs.

Panel A: Shows IL-1β release from PBMCs cultured for 48 hours with various stimuli.

Panel B: Shows parallel IL-1ra release from the same supernatants.

The data highlights the balance between IL-1ra and IL-1β production seen between the different groups.

8.3: SUMMARY

Sulphasalazine (SPZ) and its metabolites sulphapyridine (SP) and 5-aminosalicylate (ASA) have been tested for their ability to modulate T cell-, IgG- or LPS-mediated generation of IL-1β and IL-1ra by monocytes. Drug effects have been determined on healthy isolated T cells and J16 cells, as well as healthy and rheumatoid mononuclear cells and $1,25(OH)_2$-vitamin D3-differentiated THP-1 cells.

No evidence has been found for an effect of any drug, either alone or in combination, at relevant therapeutic concentrations on (anti-CD3+CD80)- or (PDBu+Ionomycin)-stimulated T cell proliferation, expression of CD69, CD28 or CD95, or the ability to stimulate cytokine production by monocytes in co-culture. Similarly, little evidence was observed for an effect of any drug on PBMC responses to LPS or IgG. The exception, however, was that SPZ (75μM) caused a marked inhibition of LPS induced IL-1β production from PBMCs purified from the blood of patients receiving long-term SPZ treatment with little effect on parallel IL-1ra production. These data suggest that SPZ may have cumulative effects on the responses of monocytes to ex vivo pro-inflammatory stimuli. Complementary to this is the observation that the profile of cytokine responses from SPZ patient PBMCs, stimulated ex vivo, favoured the production of IL-1ra over IL-1β, when compared to normal and SPRA PBMCs. In response to LPS, IgG and J16 stimulation SPZ PBMCs produced higher IL-1ra and lower IL-1β levels than any of the other groups.

It is perhaps possible that any in vitro effects of SPZ on the balance between pro- and anti-inflammatory cytokine production in normal PBMCs were not evident due to the short-term nature of such studies. The evidence from the ex vivo studies of SPZ patient PBMCs supports the already well known clinical evidence for the efficacy of SPZ in RA with data suggesting that this may be due to the balance between IL-1β and IL-1ra production.

CHAPTER 9

DISCUSSION

9: DISCUSSION

The outcome of an inflammatory reaction is controlled by a complex interaction of infiltrating immune cells, resident tissue and inflammatory cells and cytokine signalling mediators. There is a fine balance between signals that control and suppress the inflammatory response, adapting the tissue environment to one of healing and repair, and those that exacerbate the host response, eventually leading to considerable tissue damage. A breakdown in the balance of this regulation is seen in chronic inflammatory diseases such as rheumatoid arthritis, which is characterised by an inflammatory response levied against the joint tissues of the host. Whether this is as a result of an inappropriately directed antibody-driven immune response or as a consequence of an uncontrolled inflammatory incident remains to be clarified. One thing that is undisputed, however, is the important role that is played by monocyte derived cytokines in the orchestration of the events within the inflamed synovium that lead to joint destruction.

In the inflamed joint there are many possible therapeutic targets, such as the MHC molecule that is involved in antigen presentation to T cells, CD4+ T cells themselves and pro-inflammatory cytokines such as IL-1 and TNFα. Perhaps the most promising of these novel therapeutic strategies has proven to be the anti-cytokine treatments (173,175,463,464). These anti-cytokine therapies have moved with remarkable speed from the laboratory to the clinic with, in some cases, less than a decade separating the preliminary biological investigations and the present clinical usage (465,466).

In RA it is generally accepted that no one single mediator is responsible for the pathogenesis of disease, but many believe that TNFα plays a pivotal role in the generation of a pro-inflammatory cytokine profile (467,468,525). Indeed anti-TNFα therapies are proving to be clinically efficacious as are some IL-1ra and anti-IL-1β treatment strategies (463,469). Although these protein mediators are perhaps not at the apex of the initiation of RA, and as a result therapeutically targeting them will not cure disease, successful neutralisation of their effects will effectively treat the symptoms of chronic inflammation and lead to rapid disease improvement. However, despite the efficacy of the anti-cytokine therapies that is currently being demonstrated in clinical trials the question still remains as to what is driving the generation of the pro-inflammatory cytokine profile that is observed in the

rheumatoid synovium. Perhaps an even more pertinent question would be whether it is the overproduction of pro-inflammatory cytokines that is solely responsible for the pathogenesis of RA or whether it is in fact inadequate production of regulatory anti-inflammatory mediators. Our belief, which is shared by many, is that it is the balance between the production of pro- and anti-inflammatory mediators that is responsible for the pathogenesis of RA. Of particular interest to us is the balance between the production of IL-1β and IL-1ra in the rheumatoid synovium. Both of these cytokines are expressed in the rheumatoid synovium and are readily measurable in synovial fluid and in vitro cultures of dissociated synovial tissue samples. Elevated levels of IL-1ra and IL-1β are found in RA but the ratio between their concentrations is insufficient for IL-1ra to neutralise the effects of IL-1β. It is our belief that an increased understanding of factors that regulate the balance between monocyte production of IL-1β and IL-1ra would prove invaluable to the search for successful therapeutic targets for the treatment of RA.

9.1: Regulation of monocyte function in RA

Monocytes play critical roles in both the innate and adaptive immune response. In RA increased numbers of activated monocytes are observed within the inflamed synovium and lining layer and especially within the destructive area of the pannus (18,19). Blood monocytes are recruited to sites of inflammation in response to chemo-attractant gradients and interactions with activated endothelial cells that line the blood vessels. Inflammatory mediators present in the area of tissue injury induce the expression of adhesion molecules on both the monocytes and endothelium that result in cell infiltration into the inflamed joint. As monocytes and promonocytes migrate through the tissues, mediators within the inflammatory milieu can induce activation and differentiation into tissue macrophages, dendritic cells and osteoblasts (2-4,7). Thus, within the joint there is a constant flux of infiltrating cells of the monocyte/macrophage lineage as well as the already present tissue macrophages, dendritic cells and resident type A synoviocytes.

It has been demonstrated that in RA peripheral blood monocytes have increased phagocytic activity and that they secrete increased levels of pro-inflammatory mediators (20,21,23,470). Thus it is possible that the infiltrating monocytes already

display increased levels of activation prior to entering the inflamed joint due to stimulation within the periphery. Systemic activation may play an important role in RA but it is likely that the key events leading to monocyte/macrophage activation occur within the joint. Which stimulatory events are critical to the generation of a pro-inflammatory cytokine profile within the joint and especially which factors regulate the balance between monocyte production of pro- and anti-inflammatory mediators are questions that need to be answered. The studies detailed within this thesis consider mechanisms of monocyte activation that are thought to be relevant to the pathogenesis of RA. The approach that was taken was to consider factors that may be responsible for inappropriate activation of monocytes and then test how they influence the balance in monocyte cytokine production.

9.1.1: Biochemical regulation of monocyte cytokine release

In RA the environment in which the monocyte/macrophage resides is considered to be oxidative and this in itself may have grave repercussions on the state of cell activation. Reactive oxygen species such as superoxide and hydroxyl radicals generated during the respiratory burst by phagocytic cells in the area of inflammation contribute to the intracellular and extracellular reduction/oxidation (redox) balance. Intracellular redox potentials are involved in the stability of ferritin/transferrin receptor mRNAs (471) and the activation of transcription factors such as AP-1 (472) and NF-κB (473,474). More important to the studies in this thesis, however, is the effect of oxidative stress on the function of cell surface proteins such as the Na^+/K^+ATPase. The Na^+/K^+ATPase has been described as a thiol-dependent oxidation sensitive enzyme by Skou 1963 (475). Dikstein (1971) showed a correlation between the oxidation state of glutathione and the activity of Na^+/K^+ATPase with increased oxidation tending to inhibit Na^+/K^+ATPase activity (476). Further studies by Rohn et al (1993) have shown that the generation of oxygen radicals inhibits Na^+/K^+ATPase activity and that this inhibition is reversed by deferoxamine or by the addition of superoxide dismutase (477). The Na^+/K^+ATPase is a key ion-motive enzyme in the cell membrane and is one of the main mechanisms by which intracellular cation levels are regulated. Inhibition of Na^+/K^+ATPase has been shown to cause a decrease in intracellular K^+ concentration and an increase in intracellular Na^+ concentration that is linked to an increase in intracellular Ca^{2+} via

modulation of the Na^+/Ca^{2+}-exchanger (211,411). The oxidation of external thiol groups in the Na^+/K^+ATPase due to oxidative stress in the rheumatoid joint could thus lead to profound effects on intracellular cation levels.

In this thesis the specific inhibitor ouabain was used to selectively study the effect of Na^+/K^+ATPase inhibition on monocyte cytokine production. By using ouabain to inhibit the Na^+/K^+ATPase, mimicking what in the joint may be a result of oxidative damage, attempts were made to assess the role of intracellular cation levels in the regulation of monocyte cytokine production. It was found that ouabain differentially regulated cytokine production from resting peripheral blood mononuclear cells. Whilst inducing the secretion of IL-1β and to a lesser extent TNFα ouabain had no effect on IL-6 and OSM release. These results are in partial agreement with previous findings from this laboratory that demonstrated higher basal release of monocyte cytokines and more marked modulatory effects of ouabain on IL-1β, TNFα and IL-6 (232). In order to assess whether the findings shown here could be due to more quiescent mononuclear cell cultures or changes in reagents investigations were carried out to address the effects of ouabain on LPS primed PBMCs. As LPS is a potent inducer of IL-1β, TNFα, IL-6 and OSM it presented experimental conditions in which to assess whether ouabain would induce the synthesis of one or other of these cytokines. Interestingly, it was found that lower concentrations of ouabain synergised greatly with LPS in the induction of IL-1β release whilst having little effect upon TNFα, IL-6 or OSM release. At higher concentrations, however, ouabain inhibited LPS induced secretion of TNFα, IL-6 and OSM from PBMCs whilst still synergising greatly with LPS to induce IL-1β secretion. These results demonstrated that ouabain preferentially induced IL-1β secretion from resting and LPS primed PBMCs. To test whether this differential regulation of monocyte cytokine secretion would extend to modulation of anti-inflammatory cytokines the secretion of IL-1ra was measured in parallel to IL-1β. It was found that whilst synergising greatly with LPS to induce IL-1β release, ouabain markedly inhibited LPS induced secretion and intracellular accumulation of IL-1ra in PBMCs.

Inhibition of the Na^+/K^+ATPase with ouabain leads to an increase in the intracellular concentration of Na^+ and a decrease in the intracellular concentration of K^+. From these data conclusions cannot be made as to which of these cations is critical to the ability of ouabain to stimulate IL-1β synthesis from PBMCs. However, it is clear that

the intracellular concentration of these cations is in some way linked to the regulation of pro- and anti-inflammatory cytokine production from monocytes. From these results it appears that ouabain predominantly favours the production of IL-1β. These data complement previous reports that demonstrate K^+ regulatory effects on the processing and secretion of mature IL-1β (234,235,249). Perregaux et al (1992) have shown that depletion of intracellular K^+ is linked to increased cleavage of intracellular proIL-1β via modulation of the activity of ICE. Walev 1995 maintains that Na^+ and Ca^{2+} fluxes are not important to the activity of ICE but other investigators have demonstrated that both the sodium ionophore, monensin (232,478), and the calcium ionophore A23187 (233) have modulatory effects upon IL-1β secretion from monocytic cells. The data presented here cannot answer the question as to which cations are critical to the activation of ICE. It does, however, imply that inhibition of the Na^+/K^+ATPase in the rheumatoid joint due to oxidative stress could be an important factor in the generation of an imbalance between IL-1β and IL-1ra production in the synovium.

Interestingly, after the completion of these early studies, results published by Matsumori et al (1997) confirmed what had been found in our laboratory. Matsumori showed that ouabain synergised greatly with LPS to induce IL-1β secretion but had less marked effects upon IL-6 and TNFα release (479). They did not however comment upon IL-1ra production and it appears that the evidence presented here is the first to show opposing effects of ouabain upon IL-1β and IL-1ra release.

These data, however, were complicated by the observation of monocyte specific cytotoxic effects with higher concentrations of ouabain. Early studies by Walev (1995), Newton (1990) and Foey (1995), upon which these initial studies were based, and then the more recent data published by Matsumori et al. (1997) and Foey (1997) have all used ouabain at high concentrations to induce monocyte cytokine release and none have observed cytotoxic effects. It is probable that in a PBMC preparation, if death is monitored using trypan blue exclusion, that fluctuations in the viability of the monocyte population could be missed. If the sample has a low number of monocytes then the increase in stained cells due to ouabain induced death is difficult to monitor. The effects of ouabain induced death, however, may result in a profound release of IL-1β. In these studies it has been shown that concentrations of ouabain that most effectively induce IL-1β secretion from PBMCs are able to induce death of

monocytes. Death was monitored using flow cytometric scatter analysis and propidium iodide staining and apoptosis was distinguished from necrosis on the basis of phosphatidylserine externalisation. It is worth noting that IL-1β secretion was measurable several hours before any signs of monocyte death were apparent. No death was detectable until about eight hours after stimulation with ouabain although morphologically there was an apparent increase in monocyte size after three to six hours. By the twenty-four hour time-point concentrations of ouabain above 0.1μM induced death of the entire monocyte population. The activation of ICE (Caspase 1) has long been implicated in the process of apoptosis. In these studies if ouabain induced modulation of Na^+ or K^+ is responsible for the activation of ICE it is possible that both death and processed IL-1β secretion may occur depending upon which substrates are preferentially cleaved by the activated protease. This question was addressed in both purified blood monocytes and the monocytic cell line THP-1. In blood monocytes it was found that ouabain-induced secretion of mature IL-1β was accompanied by apoptotic cell death, although further experiments with more sensitive apoptosis detection systems would need to be carried out to confirm the degree of apoptosis. In contrast, however, ouabain only appeared to induce necrotic death in THP-1 cells despite secretion of IL-1β (data not shown). From these data it appears that ouabain induced activation of ICE (caspase 1) may be responsible for both the induction of IL-1β secretion and apoptosis in blood monocytes. In THP-1 cells, however, activated ICE may process proIL-1β without inducing the activation of the caspase cascade and initiating apoptosis. The absence of apoptosis in THP-1 cells may be due to the fact that they are a monocytic leukaemia-derived cell line that might not have fully functional apoptotic pathways or increased protection from molecules such as Bcl-2. From the initial findings of these studies it was considered that necrotic death, due to an osmotic stress induced by ouabain, may greatly contribute to the release of IL-1β. Recent data published by Warny and Kelly (1999), however, has linked caspase activation with necrotic death as well as apoptosis. Using the broad spectrum protease inhibitor z-VAD they demonstrated that ouabain-induced necrosis in THP-1 cells was inhibited as was IL-1β secretion (480). Also, physiological concentrations of intracellular K^+ (150mM) inhibited caspase-1 activation induced by nigericin in THP-1 cells (481). Consequently it is possible that ouabain-induced caspase activation may selectively induce IL-1β processing,

apoptosis or necrosis depending upon substrate preference or availability. It is now becoming apparent that apoptosis and necrosis may be functionally linked cell death pathways (reviewed in (482)). Both forms of cell death involve loss of mitochondrial membrane potential and osmotic homeostasis, under the control of proteins of the Bcl-2 family. It is interesting that Bcl-xL, an anti-apoptotic protein of this family, forms cation-selective membrane channels and is capable of conducting a K^+ current (483). Recently, Bcl-xL was found to inhibit necrosis caused by inhibitors of oxidative phosphorylation (484). These studies provide further evidence that intracellular cations play key roles in both apoptosis and necrosis. Elucidating the mechanism by which K^+ inhibits caspase activation will present a key step in the understanding of the regulation of cell death and its relationship with IL-1β production.

In RA it is possible that oxidative stresses within the inflamed tissues may affect other membrane proteins on infiltrating cells such as ion channels. Oxidation of K_{ATP} channels has also been shown to regulate intracellular K^+ levels in some cell types (485). In beta pancreatic cells oxidative control of K_{ATP} has been linked to membrane depolarisation, opening of voltage operated Ca^{2+} channels and subsequent regulation of insulin release (486,487). Several sulhydryl reagents have also recently been shown to induce an increase in intracellular Ca^{2+} in human T cells (488). Whether oxidative regulation of intracellular Na^+, K^+ and Ca^{2+} levels is an important factor in the generation of pro-inflammatory cytokine levels in RA remains to be determined. What has been demonstrated, however, is that intracellular Na^+ levels are increased in rheumatoid erythrocytes (412) and intracellular Ca^{2+} levels are increased in rheumatoid erythrocytes and granulocytes (489). Although not investigated here the consequence of intracellular Na^+ levels on IL-1β processing has recently been considered by Perregaux et al. (1998) (250). Previously the impaired IL-1β processing observed after complete replacement of extracellular Na^+ with K^+ was taken as evidence that increased extracellular concentrations of K^+ inhibited processing of IL-1β and linked K^+ efflux with ICE activation (235,249). Using choline- and sucrose-based minimal media, however Perregaux has now shown that extracellular Na^+ is also involved in the processing of IL-1β (250). Quite how Na^+ contributes to processing of IL-1β remains to be determined but it is possibly via an increase in intracellular Ca^{2+} due to modulation of the Na^+/Ca^{2+} exchanger. From the

analysis of monocyte responses in various cation depleted media it appears that the involvement of Na^+ in IL-1β processing precedes the activation of ICE (250). Indeed, Foey et al. have shown that the Na^+/Ca^{2+} exchanger inhibitor DCB inhibits ouabain-induced secretion of IL-1β and TNFα from resting PBMCs (232). It is apparent that modulation of intracellular cation levels by the Na^+/K^+-ATPase may have effects on several aspects of IL-1β production in monocytes. Interestingly, the Na^+/K^+-ATPase has recently been shown to differentially regulate the activation of several mitogen activated protein kinases (MAPK) (490). The two ligands, palytoxin and ouabain have been shown to compete for the same binding site and differentially regulate activation of MAPK's. Ouabain favours the activation of ERK (mitogen activated MAPK) and palytoxin favours activation of p38/JNK (stress activated MAPK). These studies, however, used ouabain at 1mM so the validity of these results might be questioned due to the osmotic stress the cells would be under. Regardless of these doubts, however, it is interesting that the Na^+/K^+-ATPase can regulate such signalling cascades and it impresses the need for more detailed study of how cation levels affect intracellular signalling processes.

In parallel studies to the investigation of ouabain effects upon human PBMC cytokine release, ouabain was also tested for pro-inflammatory effects in murine models of arthritis. Despite ouabain's ability to induce a pro-inflammatory cytokine profile in human PBMCs it was found that ouabain was unable to modulate murine antigen or zymosan induced arthritis. In the antigen induced arthritis model joint pathology was measured as bone erosion and synovitis and at no point did ouabain affect either of these scores. By utilising the more sensitive assay of proteoglycan synthesis in the zymosan induced arthritis model it was shown that ouabain could delay the recovery of joint pathology but this effect was not reproducible. It is now well reported that murine Na^+/K^+ATPase is less sensitive to the effects of ouabain than the human isoform of the enzyme. Indeed the data presented within this thesis have confirmed this using the ATPase assay to measure Na^+/K^+-ATPase activity in murine cell lines and peritoneal macrophages. Reports by Yuan et al (1993) however demonstrated long term effects upon blood pressure in rats using equivalent doses of ouabain to those used in these studies (417). Also, Matsumori et al. (1997) have recently shown that 1mg/kg ouabain given shortly before a lethal dose of LPS inhibits LPS induced death in BALB/c mice, possibly via inhibition of LPS induced IL-6 and TNFα release (479). They found that 0.1mg/kg ouabain was ineffective in

this model. Taking this evidence together it appears that some investigators have shown effects of ouabain in rodents following both long- and short-term treatment but that for short-term effects high doses of ouabain had to be used. It is thus possible that the dosing regime used in the present studies was unsuitable to assess the role of ouabain in the murine models and that the results were not a true reflection of ouabain's ability to modulate cytokine production by monocytes within the joint in vivo. It is possible that the doses of ouabain used were too low for the short-term in vivo studies and that longer pre-dosing should have been used prior to disease induction or that a much higher dose of ouabain should have been used.

Modulation of the Na^+/K^+ATPase throughout this thesis has been carried out using ouabain to mimic down-regulation of Na^+/K^+ATPase activity, which in resident cells of the rheumatoid joint may de due to oxidative damage. Another scenario that may be relevant to RA is the possibility that mononuclear cell Na^+/K^+ATPase is modulated by endogenous ouabain. Whether endogenous ouabain has any relevance to inflammatory conditions such as RA remains to be seen as most research so far has focused on cardiovascular conditions. Endogenous ouabain may well have important homeostatic functions in human biology as demonstrated by the relationship between plasma ouabain concentration and cardiac index and mean arterial pressure (248).

Although it now appears that ouabain may have very interesting effects upon cell death and that IL-1β release may be closely related to necrotic or apoptotic processes it was felt at the time of the early work that the toxic effects of ouabain were detrimental to the purpose of this research. Also, due to the slow time-course of IL-1ra release it was feared that even though ouabain might be selectively switching off IL-1ra release it was also likely that monocytes were dying before they were able to secrete significant amounts of IL-1ra. This concern, however, may indeed by an interesting facet of rheumatoid disease. Firestein et al. have described increased levels of apoptosis in rheumatoid synovial tissues (491). The primary location of apoptotic cells was the synovial lining, with the predominant cell type involved the macrophage. Apoptotic cells within the sublining layer included some macrophages and fibroblasts. If activated monocytes rapidly secrete IL-1β in response to encountering stimulation in an oxidative environment and then die before being able to secrete IL-1ra the pro-inflammatory effects of IL-1β would be unchecked. The rapid infiltration of monocytes into inflamed areas of the rheumatoid synovium

would provide a constant supply of cells that would become activated, release large quantities of IL-1β and then die.

Despite this fascinating mechanism that could be involved in the pathogenesis of RA the investigations detailed here intended to observe factors that might differentially regulate the production of IL-1β and IL-1ra. Consequently further efforts were concentrated upon other mechanisms that may be more pertinent to the selective regulation of monocyte cytokine release in RA.

9.1.2: Selective regulation between the production of IL-1ra and IL-1β from monocytes

As RA is considered an auto-immune disease it would be expected that T cells play a decisive role in both the initiation and maintenance of the disease process. Reports published by workers form the laboratory of Dayer have raised questions about the ability of T cell populations to differentially regulate monocyte production of IL-1ra and IL-1β. In the rheumatoid joint it has been shown that T cells and monocytes co-localise in areas of the pannus where great tissue destruction is occurring (345). Tissue infiltrating T cells in rheumatoid synovitis, however, do not display functional activities typically associated with an antigen driven immune response. Although the majority of the synovial T cells appear to be of the activated phenotype, as indicated by increased expression of MHC class II antigens, only a few also express the CD25 component of the high affinity IL-2 receptor (230,492). Decreased proliferative responses have also been described in rheumatoid synovial T cells (493) an observation that has also been demonstrated within this thesis as a diminished response of rheumatoid peripheral T cells to stimulation with the combination of anti-CD3 and CD80. Moreover, data on the detection of T cell-derived cytokines in rheumatoid synovial fluid and tissues are somewhat controversial. At the level of mRNA detection T cell-derived cytokines have been described by several investigators but conclusive evidence of the significant expression of cytokine protein by synovial T cells remains scarce (22,49,61,494-498). More recent data published by Steiner (1999) suggest that increased levels of T cell-derived cytokine protein are measurable in both the synovial fluid and tissue of rheumatoid patients compared to OA controls (499). These data complement cytokine secretion data from

the early description of rheumatoid synovial T cell clones in which a definite Th1 skewing was seen (500,501). There are a great deal of studies that have reported differential effects of T cell cytokines on monocyte production of IL-1β and IL-1ra. If T cell-derived cytokines reach biologically significant levels in the synovium then the predominance of Th1 type cytokines may well influence monocyte cytokine production in favour of IL-1β release.

The effect of a predominantly Th1 type T cell population in the synovium has been elegantly considered in preliminary studies by Chizzolini et al. (1997). Neglecting the importance of secreted T cell-derived cytokines they have shown that Th1 cells can preferentially induce IL-1β release and Th2 cells can preferentially induce IL-1ra release from monocytes via direct cell-cell contact alone (397). In these studies Chizzolini et al (1997) have shown that the ratio of IL-1ra to IL-1β secretion from THP-1 cells induced by contact with Th2 clones was in the range of 60:1 to 1000:1 and that induced by stimulation with Th1 clones was between 1:1 and 12:1. This T cell-derived membrane-bound signal to monocytes has been shown to involve T cell activation markers, adhesion molecules and to correlate with cell-surface expressed cytokines (354,366,368). To date, however, definitive data have not been published as to which T cell molecules are responsible for inducing monocyte cytokine secretion and more importantly how Th1 and Th2 cell populations differentially regulate the production of IL-1β and IL-1ra through membrane interactions alone.

Consequently, it was felt that further study of the ability of T cell-monocyte membrane interactions to induce differential production of IL-1β and IL-1ra could be particularly relevant to RA. It was hoped that with the clinical samples available in Bath the work of Dayer's group could be extended to study a co-culture model using rheumatoid and normal blood mononuclear cells. Looking at a co-culture model from two perspectives it would be possible to firstly study whether T cells from different patient groups could selectively regulate monocyte cytokine production and secondly, whether monocytes from different patient groups would respond differently to a control T cell stimulus.

Before a T cell/monocyte co-culture model could be established experimental conditions had to be defined for the production of IL-1β and IL-1ra from monocytes. The difference in the kinetics of monocyte IL-1β and IL-1ra release in response to

LPS and ouabain provoked further investigations into the time-course of the synthesis of these two proteins. In order to achieve such a study, different stimuli had to be selected that would efficiently induce the production of both of these cytokines from PBMCs or THP-1 cells. Based upon published work LPS and plastic-bound human IgG were utilised as suitable stimuli (502,103,229,503). Although LPS is not a factor that is considered relevant to RA it was used for its potent ability to induce IL-1β production in monocytes. LPS is also known to stimulate IL-1ra synthesis but there are less data reported that describe this function in normal human or rheumatoid PBMCs. In contrast, IgG was used for its potent ability to induce IL-1ra release from human monocytes. Binding of monocytes to plastic-bound IgG may well represent a stimulus that is relevant to RA, as FcR aggregation of a similar magnitude may be involved during clearance of immune complexes by monocytes. Using these two stimuli it was hoped that a model could be defined in which control production of IL-1ra and IL-1β could be monitored and the production of these cytokines compared in different cell populations.

It was found that in the THP-1 cell line responses to LPS were dependent upon cell surface expression of CD14. In order to increase CD14 expression THP-1 cells were differentiated with $1,25(OH)_2$-vitamin D3. All subsequent experiments used differentiated THP-1 cells (D3 THP-1) so that LPS could be used as a positive control for inducing the production of IL-1β. In contrast, IgG responses and FcR expression were comparable between resting and differentiated THP-1 cells. CD32 and CD64 but not CD16 are expressed on THP-1 cells but expression is limited to only about 15-20% of the cell population.

In normal PBMCs, purified monocytes and D3 THP-1 cells stimulation with IgG resulted in a marked release of IL-1ra with very little effect on IL-1β release. In contrast, LPS potently induced IL-1β synthesis whilst also inducing some IL-1ra production. The ability of LPS to induce IL-1ra production, however, varied greatly between cell types and individual samples. In PBMCs and purified monocytes it was found that adherence to plastic was sufficient to induce a marked release of IL-1ra. Further stimulation of adherent PBMCs or purified monocytes with LPS had variable effects upon IL-1ra release depending upon the basal level of IL-1ra release. In some patients LPS induced IL-1ra release whilst in others LPS actually suppressed basal IL-1ra release. The IL-1ra promoter has been shown to contain three LPS response

element sites that bind NF-κB (149), PU.1 (34) and GABP (34). The crystal structures of these transcription factors suggest that they will not bind together and will compete for occupancy (151-154). It is thus possible that LPS induced signals will inhibit adherence induced signals via competition for binding sites on the IL-1ra promoter. In contrast, D3 THP-1 cells produced very little IL-1ra in resting culture, perhaps as a result of their inability to adhere to plastic. LPS stimulation of D3 THP-1 cells produced a concentration dependent increase in IL-1ra release. The kinetics of IL-1β and IL-1ra production was quite different regardless of cell type or stimulus. IL-1β was measurable much earlier than IL-1ra and levels secreted reached a plateaux between twenty-four and forty-eight hours after stimulation. In contrast, the production of IL-1ra climbed steadily even after seventy-two hours of culture. Consequently, a forty-eight hour time-point was used in all further experiments in order to be able to compare the levels of IL-1β and IL-1ra release. The main query with such an experimental design is that between cell stimulation and cytokine determination there is a large window for complications such as feedback regulation, which may not be stimulus-specific. For example the varying effects of LPS on IL-1ra release seen in these studies and the delayed appearance of IL-1ra might suggest that IL-1ra production is induced by feedback effects of LPS induced IL-1β. Considering this however, as the hope was to compare stimuli that are relevant to the setting of the rheumatoid joint it was felt that such non-specific feedback mechanisms, which may be beyond basic experimental control, would not detract from the information obtained from these studies. If, for example, LPS induced IL-1ra was in fact an artefact of feedback effects of IL-1β this would easily be confirmed using neutralising antibodies against IL-1β.

It has been demonstrated that for IL-1ra to effectively inhibit the biological activity of IL-1β that a ratio of IL-1ra:IL-1β of at least 100:1 needs to exist. Applying this criterion to these studies helps to highlight the biological significance of the stimuli being studied. For example in resting (cultured on plastic) PBMCs and purified monocytes a ratio of IL-1ra:IL-1β of 100:1 and 700:1 respectively is achieved after forty-eight hours of culture. In both these cell types stimulation with LPS reduces this ratio to 4:1 indicating the probable physiological dominance of IL-1β under these conditions. In contrast, although stimulation with IgG was seen to induce IL-1β secretion the excess of IL-1ra production maintains the ratio at 120:1 in PBMCs and

250:1 in purified monocytes. Very similar results are seen in D3 THP-1 cells, with LPS and IgG stimulation inducing an IL-1ra:IL-1β ratio of 3:1 and 133:1 respectively. Thus from these studies there is no evidence to support a pro-inflammatory role for stimulation of monocytes through FcR aggregation, in terms of the balance between IL-1β and IL-1ra release, and there is no evidence that LPS induced secretion of IL-1ra will have biological significance.

The ability of FcR aggregation to induce monocyte IL-1ra production questions whether immune complexes are in fact a pro-inflammatory stimulus in RA. Larger immune complexes bind complement and perhaps this cascade is more important for inflammatory responses to immune complexes in RA. Monocytic binding and phagocytosis of immune complexes via FcR interactions may well, however, involve the induction of IL-1ra release that protects the local environment from an inappropriate inflammatory response. Such a mechanism would allow the safe clearance of immune complexes in the absence of tissue damage.

9.1.3: T cell driven monocyte cytokine production

Having established control responses for the production of IL-1β and IL-1ra from monocytes these studies were extended to examine the effects of interactions with T cells on monocyte cytokine secretion. A similar model has already been described by Vey et al. (1992) using various T cell lines and THP-1 cells (352). The studies described here, however, were intended to extend the application of such a model to the study of clinical samples from RA patients. It was also felt that a co-culture model would be pertinent for the study of an anti-rheumatic compound such as sulphasalazine (SPZ), which has significant but as yet undefined clinical efficacy. Consequently studies were aimed at showing whether the current monocytic cultures would produce IL-1β and IL-1ra in response to T cells, whether such a stimulus would selectively induce the production of one of these cytokines and whether the balance in the production of IL-1β and IL-1ra would be influenced by SPZ.

Using fixed resting or PDBu/ionomycin activated J16 cells it was shown that direct cell-cell contact was sufficient to induce IL-1β and IL-1ra production from D3 THP-1 cells, PBMCs and purified monocytes. The ratio between IL-1β and IL-1ra production from all three of the monocytic cell types studied varied depending upon

the activation state of the J16 cells. In D3 THP-1 cells, resting and activated J16 cells induced a significant release of IL-1ra but only activated J16 cells had the ability to induce IL-1β secretion. Comparing these effects in respect to the levels of IL-1ra to IL-1β produced, a ratio of 60:1 was observed when D3 THP-1 cells were stimulated with activated J16 cells. Thus it is apparent that even though activated J16 cells induce the secretion of IL-1β from D3 THP-1 cells the parallel release of IL-1ra is perhaps sufficient to counteract any biological effects of such stimulation. In PBMCs and purified monocytes the results of co-culture with J16 cells are somewhat complicated. Resting and activated J16 cells stimulated both IL-1β and IL-1ra from these cell types. Resting J16 cells induced a cytokine profile that favoured biological effects of IL-1β but activated J16 cells increased the ratio in favour of IL-1ra, especially in purified monocytes. Despite this slight variation in responsiveness to J16 cells, however, it was interesting to note that PBMC responses were broadly very similar to those of purified monocytes. These results thus question the role of the autologous T cell population that is present in the PBMC preparation. In comparison to IgG and LPS induced monocyte responses it was found that activated J16 cells induced a similar cytokine profile to that of IgG stimulation, with IL-1ra production being favoured over that of IL-1β whereas LPS favoured the production of IL-1β.

As D3 THP-1 cells secrete very low levels of both IL-1β and IL-1ra under basal conditions they are an appropriate model monocyte for the determination of stimuli that induce the production of both IL-1ra and IL-1β. It was found that the ability of J16 cells to induce cytokine release from the D3 THP-1 cells was strongly influenced by their activation state. J16 cells proliferate at very high rates under normal culture conditions and upon stimulation with phorbol ester + ionomycin or anti-CD3 + CD80 are seen to stop multiplying. Consequently, the activation of J16 cells was determined via surface staining of activation markers. The ability of J16 cells to induce cytokine production from D3 THP-1 cells was related to the level of CD69 expression. The ability of activated peripheral blood T cells to induce D3 THP-1 IL-1ra release was also seen to correlate with CD69 expression. These data as well as reports in the literature that anti-CD69 neutralising antibodies could block T cell induced secretion of cytokines from monocytes (354,366,368) prompted further investigations into the role of CD69 in this model. As there is no evidence to identify the monocyte ligand for T cell expressed CD69 there were not many further

experiments that could be conducted to see what the involvement of CD69 was in the co-culture model. Neutralising antibody experiments were felt to have an element of doubt due to the fact that the binding of an antibody molecule to the cell surface may also inhibit the interactions of neighbouring molecules. Also, in light of the ability of FcR aggregation in monocytes to induce the production of IL-1ra it is possible that presenting the monocyte with an activated T cell, that is covered in anti-CD69 IgG, could itself induce activation. Consequently, CD69 was cloned and expressed in the COS cell line so that a positive response to CD69 could be looked for instead of a tool to inhibit a response. Preliminary data from this work support a role for CD69 in stimulating monocyte production of IL-1β. The COS cells alone, however, induced the production of both IL-1β and IL-1ra from D3 THP-1 cells suggesting that the monkey derived cell line may possess surface markers with significant homology to human adhesion molecules. In these studies, however, CD69 expressing COS cells induced greater IL-1β secretion from D3 THP-1 cells and this reached significant levels when the D3 THP-1 cells were co-stimulated with LPS. These data do not prove that T cell CD69 is entirely responsible for the ability of activated T cells to induce monocyte cytokine secretion but they do show a contributory role.

In a study using normal purified T cells that were activated, fixed and then re-cultured with autologous purified monocytes, similar effects were seen to those studies using J16 cells. The ability of activated T cells to induce monocyte IL-1β production correlated with the ability of the activation stimulus to induce T cell proliferation. Both resting and activated T cells, however were able to stimulate marked IL-1ra release from monocytes but the differences were marginal. In fact, the release of IL-1ra from monocytes co-cultured with resting T cells was so high that significant effects of any of the activated T cell groups were hard to define. Interestingly, it has been suggested recently that rheumatoid synovial T cells that are anergic due to inappropriate antigen presentation in the synovium may have the capacity to modulate monocyte cytokine production despite their inability to proliferate (Panayi, personal communication). The data presented here, however, suggest that cells that proliferate in response to stimuli, in this case anti-CD3 and CD80, express a surface phenotype that can interact with monocytes and induce the secretion of IL-1β and IL-1ra. Thus it is possible that the ability of T cells to drive monocyte cytokine release is more closely related to the state of cell activation and

not necessarily their ability to proliferate. In their resting state fixed T cells induce a very high level of IL-1ra release from monocytes with very little IL-1β release. Upon activation fixed T cells in this study did not develop the ability to significantly raise IL-1β release in relation to IL-1ra release. Consequently it may be surmised that factors other than membrane interactions alone are likely to be required for T cells to be able to induce a pro-inflammatory level of IL-1β secretion from monocytes in the rheumatoid synovium.

T cell-derived cytokines clearly have regulatory roles on the secretion of IL-1β and IL-1ra from stimulated monocytes. As the detection of significant levels of T cell-derived cytokines in rheumatoid synovial fluid is not universally accepted the initial studies detailed here were based upon the reports of T cell membrane-restricted stimulation of monocytes (351,353,354,366,368,381). Consequently, all co-culture studies described so far have used fixed T cells or J16 cells to stimulate monocyte cytokine release. To investigate the contribution of T cell-derived soluble factors in T cell stimulated monocyte cytokine release co-culture assays were established using unfixed cells. Unfixed resting J16 cells induced significantly less IL-1ra release from D3 THP-1 cells than fixed J16 cells. In contrast, unfixed J16 cells induced significantly more IL-1β release from D3 THP-1 cells than fixed J16 cells. Activation of the J16 cells had no effect on their ability to modulate the balance between IL-1ra and IL-1β production from D3 THP-1 cells although the actual levels of cytokine released increased. When J16 cells were used to stimulate cytokine release from PBMCs it was again found that unfixed J16 cells induced a much greater production of IL-1β than fixed J16 cells. The effect of fixation on the ability of J16 cells to induced IL-1ra release from PBMCs was masked by the already very high basal release of IL-1ra. These data suggest that in this model J16 cell contact dependent stimuli preferentially induce IL-1ra release from D3 THP-1 cells and soluble factors override this to favour IL-1β release from both D3 THP-1 cells and PBMCs. Interestingly, the addition of LPS to this system had profound synergistic effects on the ability of J16 cells to induce IL-1β secretion from D3 THP-1 cells and PBMCs. In particular, stimulation of D3 THP-1 with unfixed activated J16 cells markedly synergised with LPS to induce the secretion of IL-1β. In contrast, the combination of J16 cell and IgG stimulation only had an additive effect upon both IL-1ra and IL-1β production by D3 THP-1 cells. In PBMCs a similar degree of

synergy was seen when unfixed J16 cells and LPS were used in combination to stimulate IL-1β release, although in this case the effect was not dependent upon activation of the J16 cells. A very similar synergy was observed when activated peripheral T cells and LPS were used to stimulate IL-1β production from D3 THP-1 cells. In this case, however, the degree of synergistic IL-1β production induced was dependent upon the stage of T cell activation and the activating stimulus that had been used. In particular, T cells that had been activated with SEA for six to seven days consistently synergised most potently with LPS to induce IL-1β secretion from D3 THP-1 cells. Based upon such low experimental numbers it is difficult to be sure of this response. Also, there was no correlation with T cell surface marker expression suggesting the possibility that the synergy with LPS was due to a soluble factor. Further work is in progress to assess the ability of LPS to stimulate soluble mediator production from various T cell populations. This synergy between live unfixed T cells and LPS in stimulating IL-1β release from monocytic cells may not be immediately relevant to rheumatoid disease but it does highlight that multiple signals may converge on the monocyte to induce a marked shift in the balance between IL-1ra and IL-1β secretion. Extrapolating these data, however, it might be possible that other mediators that are more relevant to RA, such as TNFα, may have the ability to synergise with T cell stimulation of monocytes. From these data it is difficult to say whether the monocyte is synergistically responding to distinct signals, one from the LPS and one from the T cell membrane with or without a contribution from soluble mediators, or whether the LPS could be modulating the T cell directly. The effects of LPS on T cell responses are not well documented. Tennenberg and Wellers (1996), however, described the ability of LPS to induce T cell IFNγ production in the presence of endothelium (504) and more recently Mattern et al have shown that LPS and its lipid A component are potent inducers of T cell proliferation and of Th1-type cytokine production (505). Further studies by Mattern et al have also shown that the ability of LPS to drive T cell proliferation is dependent upon the presence of viable monocytes, is MHC unrestricted but is dependent upon CD80 co-stimulation (506). These reports suggest that the synergistic induction of monocyte IL-1β secretion might be due to reciprocal activation of the T cells by LPS and monocyte contact. To test whether LPS alone might induce the production of pro-inflammatory cytokines from activated J16 cells preliminary experiments have been carried out by

transferring stimulated J16 supernatants to culture with purified monocytes (data not shown). It has been found that when activated J16 cells are stimulated with LPS they produce a soluble factor that induces monocyte IL-1β secretion. The level of IL-1β secretion induced in monocytes by the conditioned J16 medium has been found to be markedly higher than that induced by the concentration of LPS relevant to that carried over from the J16 culture.

Interestingly, sub-toxic concentrations of ouabain were also able to synergise with unfixed J6 cell stimulation of IL-1β production from PBMCs. Ouabain at 1-10nM had no effect on fixed J16 cell induced IL-1β secretion from PBMCs. These data suggest that the ability of ouabain to modulate T cell driven secretion of IL-1β from monocytes is dependent upon T cell viability. Whether this is due to the release of a T cell factor in response to ouabain or the synergy between an already present T cell factor and the ability of ouabain to modulate the activity of ICE in monocytes remains to be determined. The ability of ouabain to modulate T cell activation is a subject that has not been investigated here and is perhaps an important area that could bring together important features of RA pathology. Oxidative damage of the Na^+/K^+ATPase in both synovial lymphocytes and monocytes may well contribute to the development of a pro-inflammatory cytokine profile.

The identity of a candidate T cell-derived factor whose production is induced by LPS remains to be determined. The report of LPS induced IFNγ production from T cells (504), however, would fit with this model as IFNγ has been shown to up-regulate LPS induced IL-1β release from monocytes (349,507-509). IFNγ has recently been shown to potently augment activation of NF-κB in monocytes stimulated with LPS and to accelerate binding of NF-κB to DNA (510). In this same study Held et al (1999) also showed that LPS enhances the activation of signal-transducing activator of transcription-1 (STAT1) induced by IFNγ. These data show that in monocytes distinct stimuli may mutually potentiate their individual effects. Other possible T cell-derived soluble factor candidates include IL-17 and monocyte migration inhibitory factor (MIF). Both of these have been shown to induce monocyte IL-1β secretion alone or in combination with other stimuli (511,512). As LPS is a potent inducer of MIF secretion from monocytes, and MIF plays an important role in T cell proliferation it is possible that the LPS induced proliferation of T cells described by

Matern et al. (1998) (506) might involve MIF secretion. Tetanus toxoid driven proliferation of T cells in the presence of autologous monocytes has been shown to be inhibited by anti-MIF antibodies (513). Whether MIF is involved in the synergy between LPS and viable T cell-derived stimulation of monocyte IL-1β secretion remains to be determined. It is possible that MIF plays an important role in regulation of both monocytes and T cells in the rheumatoid joint, however, as levels measured in synovial fluid of RA patients are greatly elevated (514).

Taken together, the co-culture data shown here support a role for both T cell-derived soluble mediators and membrane bound molecules in the induction of monocyte cytokine production in RA. The T cell dependence of monocyte activation in the rheumatoid synovium has recently received further support from Klimiuk et al. (1999) (515). They have shown, using adoptive transfer or depletion of T cells into rheumatoid synovial membrane engrafted NOD-SCID mice, that the production of IL-1β, TNFα and MMPS is T cell dependent. In this thesis it has been shown that resting and activated J16 cells or blood T cells are capable of inducing production of IL-1β and IL-1ra from PBMCs, purified monocytes and D3 THP-1 cells. Questions still remain as to the specific interactions involved but despite this the T cell/monocyte co-culture model appears to be sufficiently well characterised for the study of modulatory compounds or clinical samples. Unfortunately, due to time constraints this model was not used to investigate the ability of rheumatoid T cells to induce D3 THP-1 cytokine production. It is felt, however that this would be a very fruitful study in which it would be possible to compare the ability of peripheral and synovial T cells from various patient groups for their ability to induce changes in the balance of IL-1ra and IL-1β production from D3 THP-1 cells. If the data published by Chizzolini (1997) using T cell clones holds to be true then it would be expected that normal T cells of the Th1 phenotype would also favour the induction of IL-1β secretion from D3 THP-1 cells. Comparing the clinical samples to normal healthy samples it would be interesting to see if increased Th1-like activity could be demonstrated using monocyte cytokines as a measurable outcome. D3 THP-1 cell co-culture may offer a convenient model to show whether the predominance of Th1-like activity is confined to synovial T cells in RA thus backing up evidence of Th1 cytokine profiles in synovial fluid.

The co-culture model was used, however, to study the ability of sulphasalazine (SPZ) to modulate T cell driven cytokine production from monocytes. In these studies sulphasalazine and its major metabolites were assessed for their ability to modulate T cell proliferation and T cell activation marker expression. Also, the test compounds were assessed for their ability to modulate control monocyte responses to IgG and LPS. Finally, using the J16 cell line the test compounds were assessed for their ability to modulate fixed J16 cell induced IL-1β and IL-1ra secretion from D3 THP-1 cells, normal PBMCs, chronic rheumatoid PBMCs and SPZ treated rheumatoid PBMCs. Neither SPZ or its major metabolites significantly modulated any of the responses measured in these studies. It became apparent, however, that the PBMCs from the SPZ patients showed a general profile of cytokine release in response to all stimuli tested in which they produced higher levels of IL-1ra and lower levels of IL-1β than any of the other sample groups. Unfortunately this finding is as yet statistically insignificant but further studies are underway to improve these data. The possibility that SPZ is regulating patient PBMC cytokine secretion could be due to long-term protection of monocytes from oxidative damage. SPZ has been shown to scavenge ROI produced by neutrophils, monocytes and macrophages (516). By protecting cells from oxidative damage SPZ may inhibit damage to surface proteins such as K_{ATP} or Na^+/K^+-ATPase. From the data presented here it can be surmised that protection of monocyte Na^+/K^+-ATPase from ROI induced inhibition could regulate intracellular cation levels and subsequently attenuate IL-1β secretion. As well as ROI scavenging SPZ has been shown to inhibit LPS induced IL-1β, IL-6 and TNFα from monocytes in vitro (517) and to also inhibit IL-2 secretion from human T cells (518,519) possibly via inhibition of NF-κB (520). These data support the ability of SPZ to affect both T cell and monocyte responses and therefore support the use of the co-culture model for the analysis of mechanisms of action of SPZ. In some of these reports, however, concentrations of SPZ used far exceeded the mean therapeutic levels found in patient serum with levels that were between 50- and 100-fold higher being used. This fact, combined with the results presented here, suggest that although SPZ remains a drug with valuable clinical efficacy in RA it does not appear to be able to modulate monocyte cytokine release in short term in vitro assays. The much more promising data, which is as yet still statistically insignificant, is that long-term treatment with SPZ may well modulate monocyte cytokine production in such a way as to favour IL-1ra production above that of IL-1β. The mechanism by which SPZ

might be able modulate monocyte cytokine production remains to be determined. In studies by Vey et al (1997) the induction of IL-1β and IL-1ra secretion in THP-1 cells was shown to be differentially regulated by the serine/threonine phosphatase inhibitor okadaic acid (381). Inhibition of serine/threonine phosphatases was shown to have no effect on basal release of IL-1β and IL-1ra but was shown to up-regulate IL-1β secretion from T cell stimulated THP-1 cells. In the preliminary studies detailed here it has also been shown that PI3-K may play an important role in IL-1ra production but not IL-1β production by PBMCs and D3 THP-1 cells in response to stimulation with LPS, IgG or activated T cells. The selective PI3-K inhibitor LY 294002 was shown to potently inhibit IL-1ra secretion from monocytic cells but had no effect on IL-1β secretion in response to any stimuli studied. Although preliminary, these data suggest an important role for PI3-K in the regulation of IL-1ra release from both resting and stimulated monocytes. Whether SPZ could potentiate signalling through PI3-K or serine/threonine phosphatases remains to be determined.

9.2: SUMMARY AND CONCLUSIONS

The studies of this thesis have shown that the production of cytokines from monocytes, particularly IL-1β and IL-1ra are differentially sensitive to stimuli such as LPS, IgG, T cells and cation modulation. Inhibition of the Na^+/K^+-ATPase, which in the synovium may be due to oxidative damage, has been shown to greatly contribute to the generation of a pro-inflammatory cytokine profile from stimulated monocytic cells. The specific Na^+/K^+-ATPase inhibitor ouabain has been shown to increase IL-1β processing in monocytes stimulated with LPS, or viable activated T cells, and to concurrently down-regulate IL-1ra production. The differential response of normal, early rheumatoid and chronic rheumatoid patient PBMCs to stimulation with ouabain alone may simply reflect increased processing of an already present pool of proIL-1β rather than differential sensitivities to ouabain. The mechanism of action of ouabain in these models may well involve increased activation of ICE due to K^+ efflux and indeed it has been shown that increased concentrations of ouabain induce a toxic reaction in monocytes that may well involve activation of ICE-like caspases.

The ability of T cells to regulate production of IL-1β and IL-1ra from neighbouring monocytes may be an important mechanism in the pathogenesis of RA. The studies here have shown the successful development of a co-culture model in which to assess the contribution of different T cell populations to monocyte cytokine production. This model will allow improved study of different patient T cell and monocyte responses and will be ideal for the study of potential anti-inflammatory compounds. The ability of monocytes to secrete IL-1ra under resting conditions highlights the regulatory role of this cytokine in the prevention of an inflammatory reaction. The models studied here have shown that fixed T cells were able to induce the secretion of both IL-1β and IL-1ra from resting monocytes, perhaps due to surface CD69 expression, but that the balance in cytokine production was not consistently driven in a pro- or anti-inflammatory direction. Stimulating the monocytes with viable T cells, LPS or a combination of both, however, resulted in a marked swing in the balance of cytokine production to favour biological effects of IL-1β. From these data it can thus be concluded that T cell membrane derived signals are involved in the induction of monocyte cytokine secretion but that soluble factors are critical to the development of a pro-inflammatory response.

9.3: FUTURE WORK

The studies detailed here have asked almost as many questions as they have answered. As a result there are many avenues of investigation that are now open for continued study. Looking back on the Na^+/K^+ATPase studies in light of the more recent data published on the involvement of Caspase-1 (ICE) in both apoptosis and necrosis it would be interesting to pursue a more detailed study of the ability of ouabain to induce apoptosis. If indeed early death of monocytes occurs in the rheumatoid joint and involves K^+ regulation of Caspase-1 then oxidative damage of ion channels or Na^+/K^+ATPase may well be critical events in the pathogenesis of RA. Similarly the presence of endogenous ouabain in synovial fluid might play a role in inducing death of monocytes and inducing a pro-inflammatory balance in the production of IL-1β and IL-1ra.

The development of the T cell/monocyte co-culture model that has been established here also opens many opportunities for further studies. Of immediate interest would

be the comparison of rheumatoid synovial and peripheral T cells for their ability to induce D3 THP-1 cytokine secretion. Combined with this there is a great need to further investigate whether membrane molecules and surface bound cytokines or T cell-derived soluble factors are capable of selectively inducing the production of IL-1β or IL-1ra.

Also, the promising data on the ability of SPZ treatment to favour monocyte production of IL-1ra above that of IL-1β needs to be clarified for publication.

APPENDIX 1

APPENDIX 1

GROWTH MEDIUM, BUFFERS AND SOLUTIONS

1: Dulbecco's Minimal Essential Medium (DMEM)

1.1: Glutamine free DMEM for CHO Cells

DMEM (1X)	500mls
FCS	55mls
Penicillin (1000IU/ml)/ Streptomycin (1000µg/ml)	5mls
Nucleosides (100X)	5mls

Nucleosides (100X)	Thymidine (Sigma)	0.34mg/ml
	Guanosine (Sigma)	0.7mg/ml
	Adenosine (Sigma)	0.7mg/ml
	Cytidine (Sigma)	0.7mg/ml

1.2: DMEM for COS Cells

DMEM was prepared as above with the addition of 5ml L-glutamine (200mM stock).

1.3: DMEM for Murine Patellae culture

DMEM was prepared as above except 0.1% BSA (w/v) was used instead of FCS. 1.85 MBq ^{35}S [Na_2SO_4] (Amersham, UK) was added for the last three hours of culture.

2: RPMI-1640 for all T cell, PBMC, J16, THP-1 and murine monocyte culture

Milli-Q water	400mls
RPMI (10X)	55mls
FCS	50mls
Penicillin (1000IU/ml)/ Streptomycin (1000µg/ml)	5mls
Sodium Bicarbonate (7.5% w/v)	15mls
Glutamine (200mM)	5mls
Sodium hydroxide (10M)	750µl

3: Phosphate Buffered Saline (PBS)

Five tablets (Oxoid) added to 500mls of milli-Q water and sterilized by autoclave.

4: ELISA solutions

Coating buffer	0.1M bicarbonate, pH9.6
Blocking buffer	1% BSA, 5% sucrose in PBS, pH7.4
Wash buffer	0.05% Tween 20 in PBS, pH7.4
Diluent	0.05% Tween 20, 0.1%BSA in Tris Saline pH7.3 (20mM Trizma base,150mM NaCl)
Stop solution	0.5M H_2SO_4

5: ATPase Assay Cocktail

The assay cocktail was composed of the following components, dissolved in milli-Q water and pH adjusted to 7.4:

NaCl	100 mM
KCl	20 mM
$MgSO_4$	4.5 mM
EGTA	5 mM
Na_2ATP	3 mM
PEP	1.2 mM
Trimethylamino-ethanesulphonic acid-Tris buffer (TES)	40 mM

6: Cell Lysis Buffer

The following components dissolved in milli Q water and stored at 4°C.

Hepes	10mM
$MgCl_2$	1.5mM
KCl	10mM
NP-40	0.2%
To this stock the following were added on the day of use:	
DTT	1mM
PMSF	0.2mM
1ug/ml Pepstatin A	1:1000 of stock (1mg/ml)
5ug/ml Leupeptin	1:1000 of stock (5mg/ml)

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